Anthraquinone-Peptide Conjugates As Inhibitors Of DNA Transcription Factor Binding

By

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A Thesis Submitted to De Montfort University in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy

November 1998

DECLARATION

The research work reported in this thesis was carried out between October 1994 and December 1997, and unless otherwise accredited, the work was carried out by the author. No portion of the work referred to in this thesis has been submitted in support of an application for any other degree or qualification at De Montfort University or any other institute of higher education.

Taeeba Ijaz

Dedicated to Dad, Mum, Baj, Farah, Amer and Maryam

ACKNOWLEDGEMENTS

A very special thank you to my supervisors Professor Laurence Patterson and Dr Paul Teesdale-Spittle for all their help, support and encouragement. I would also like to thank John Lamb for providing the mass spectra data, Norma Garrington for running the IR spectra, Dr Parvez Haris for providing the Circular Dichroism data and Phoung Tran for conducting the EMSA. Many thanks to Dr Mike Needham for running the NMR experiments and for all his time and helpful advice. Thanks to all the technical and academic staff and to my research colleagues, both past and present, for all their help and support. I would also like to take this opportunity to thank the Association of International Cancer Research Campaign for funding this project.

Also a very big thank you to Ketan and Ria for all their assistance and encouragement and particularly to Linda, Rehana and Sims for being such good friends and colleagues and, more importantly, for keeping me sane! And finally, a gigantic thank you to all my family and friends for all their support, encouragement and patience throughout my work!

ABSTRACT

Inhibitors of Activator Protein-1 (AP-1), a collective term referring to dimeric transcription factors involved in mediating long term responses to signals that regulate growth control and development, were designed and synthesised in this study. Truncated peptide sequences of 5-7 residues containing the highly conserved tri-amino acid sequence KCR found in the DNA binding domains of the AP-1 family of proteins were attached to mono-substituted anthraquinones through various amino acid type linkers. It was envisaged that these intercalator-linked peptides would have the advantage of high DNA binding with some sequence selectivity.

The peptides (1:ARCKA; 2:AKCRA; 3:AKSRA; 4:AKCRNA; 5:AKCRKA; 6:AKCRNKA; 7:AKCRKRA; 8:AAKCRAA) were synthesised using solid phase peptide synthesis. The amino acid type linkers were attached to 1-chloroanthraquinone via a nucleophilic displacement reaction and subsequently coupled to the N-terminus of the resin bound peptides using PyBOP. Characterisation of the peptides and the intercalator-linked peptides was conducted using HPLC, MALDI-MS and 2D-NMR spectroscopy. Biophysical analysis of the peptide conjugates with DNA using fluorimetry and thermal denaturation studies indicated that the AP-1 inhibitors were weak intercalators of DNA and suggested that DNA binding of these intercalator-linked peptides was governed, in principle, by the peptidic moiety. Electrophoretic mobility shift assays of the peptides and their respective conjugates demonstrated that the latter were more effective in displacing the AP-1 protein compared to the peptide moieties alone. These assays also showed that the peptides and conjugates bearing the sequences AKCRNA (peptide 4) and AKCRKRA (peptide 7) were particularly effective in inhibiting the AP-1 protein binding. Peptides 4 and 7 were also shown to possess an intrinsic propensity to form helical structures upon transfer to hydrophobic environments and also to interact with the DNA AP-1 consensus sequence more favourably compared to peptides 1, 2, 3, 5, 6 and 8 in the circular dichroism and molecular modelling studies respectively.

ABBREVIATIONS

Ala (A)	Alanine
Arg (R)	Arginine
Asn (N)	Asparagine
AP-1	Activator Protein-1
ATF	Activating Transcription Factor
Boc	t-Butyloxycarbonyl
BOP	Benzotriazole-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate
CD	Circular Dichroism
COSY	Correlated Spectroscopy
CRE	Cyclic Adenosine Monophosphate Response Element
Cys (C)	Cysteine
DIEA	Diisopropylethylamine
DKP	2,5-Diketopiperazine
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDT	Ethanedithiol
EMSA	Electrophoretic Mobility Shift Assay

Fmoc	9-Fluorenylmethoxycarbonyl
GABA	γ-Aminobutyric acid
HOBt	N-Hydroxybenzotriazole
HPLC	High Performance Liquid Chromatography
IR	Infrared
Lys (K)	Lysine
MALDI-MS	Matrix Assisted Laser Adsorption Ionisation Mass Spectrometer
Mtr	4-Methoxy-2,3,6-trimethylbenzenesulphonyl
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Enhancement Spectroscopy
ODmab	4-{N-[1-(4,4-Dimethyl-2,6-dioxocyclohexlidene)-3-methylbutyl]amino}benzyl
Opfp	Pentafluorophenyl ester
Pbf	2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulphonyl
Pmc	2,2,5,7,8-Pentamethylchroman-6-sulphonyl
PyBOP	Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexa-fluorophosphate
Ref-1	Redox Factor
Ser (S)	Serine
SPPS	Solid Phase Peptide Synthesis
TFA	Trifluoroacetic acid
TFE	Trifluoroethanol

TIS	Triisopropylsilane
TLC	Thin Layer Chromatography
TRE	12-O-tetradecanoylphorbol-13-acetate Response Element
Trt	Trityl
UV	Ultra Violet light
VIS	Visible light

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Chapter Three

1 INTRODUCTION

1.1 Cancer

Cancer is a complex family of diseases that results from the breakdown or suppression of the mechanisms responsible for normal cellular growth and development. The relentless unchecked division of cancer cells results in the formation of malignant tumours that compress and crowd out surrounding healthy tissue. As tumour growth progresses, angiogenesis takes place, where upon a tumour vasculature system develops to provide oxygen and other metabolites to nourish the rapidly expanding cell mass [1,2]. The vasculature system, however is often abnormal or poorly developed which means that the blood flow and rates of diffusion to these tumours is not uniform. This results in insufficient nutrient delivery to the tumour cells, which become starved of oxygen and metabolites and therefore become necrotic or hypoxic [3].

1.2 Hypoxic Tumours

Oxygen deficient (i.e. hypoxic) cells represent a limiting factor for the therapeutic treatment of solid tumours (figure 1) due to increased radiation and cytotoxic chemotherapy resistance relative to oxygenated tissues [4]. These cells are approximately three times less sensitive to X-irradiation, a condition resulting from the diminished formation and altered half times of reactive oxygen radicals in a hypoxic environment [5,6]. The same is true of cytotoxic agents that require the formation of radicals within the cell. Resistance to anticancer agents in solid

tumours is also a direct result of decreasing transport of drugs to the cells as they become increasingly distant from operative vasculature systems as tumour growth progresses [7]. Solid tumours, in particular those that are under oxidative stress pose a serious problem in the effective treatment of cancer [8].

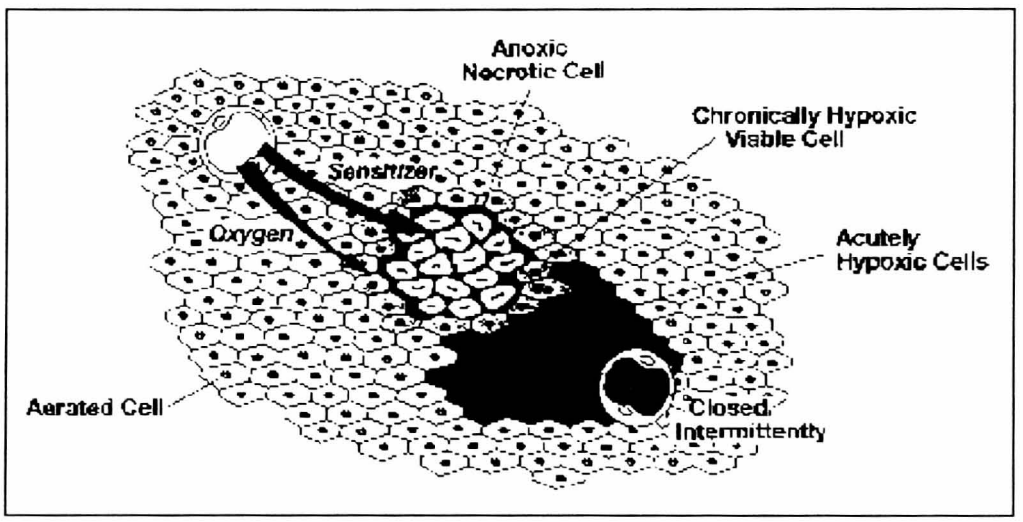


Figure 1 Schematic Representation of a Hypoxic Tumour (the area in black represents acute hypoxia) [9]

Another important feature of hypoxic tumours is their ability to continue growing in the presence of very little or no oxygen. This observation has been attributed to the fact that the affinity of various transcription factors involved in stress responses for their cognate DNA sequences is dependent on the redox state of specific cysteine (Cys (C)) residues found in the DNA binding domains of these proteins [10,11]. Such transcription factors include the Myb, NF-κB proteins and in particular the activator protein-1 (AP-1), all of which become active under

reducing conditions, including hypoxia, thus permitting transcription and hence tumour growth [12,13]. The activation of the AP-1 under reducing conditions and its role in oncogenesis is discussed in section 1.3.4.

1.3 Activator Protein-1

AP-1 is a collective term referring to dimeric transcription factors composed of subunits from the fos, jun and activating transcription factor (ATF) multigene families. The fos family contains four proteins (c-fos, fos-B, fra-1, fra-2) while the jun and ATF families are each composed of three (c-jun, jun-B, jun-D and ATF-2, ATF3/LRF1, B-ATF respectively) [14]. The AP-1 consists of homodimers and heterodimers of the fos, jun and ATF proteins which have the common property of interacting with the DNA sequence, TGACTCA, known as the AP-1 binding site or the 12-O-tetradecanoylphorbol-13-acetate (TPA) responsive element (TRE) and also to certain closely related variants such as the TGACTGCA sequence, referred to as the cyclic adenosine monophosphate (cAMP) response element (CRE) [10,12]. These transcription factors are involved in the activation of several genes including those coding for metallothionein II_A, collagenase and stromelysin [10]. There are also binding sites for the AP-1 in the simian virus 40 enhancer regions as well as in numerous other cellular genes including the DT-diaphorase and glutathione S-transferase genes [15,16]. The AP-1 is therefore a sequence specific protein which is involved in mediating long-term responses to signals that regulate growth control and development [11,12].

Fos and jun, like the ATF/CREB and GCN4 proteins are members of the bZip family of sequence specific dimeric DNA binding proteins. The members of this family share a common structural motif, but differ in their ability to differentiate DNA target sites [17]. The term 'bZip' refers to the amino acid sequences of two independently acting sub-regions (figure 2) of the DNA binding domain. One region facilitates dimer formation and the other contacts DNA. The dimer-forming region, termed the leucine zipper (Zip) is characterised by a pattern of leucine (Leu) residues repeating every seventh amino acid and is responsible for mediating protein-protein interactions [18]. At the NH_2-terminal of the leucine zipper region there is a basic (b) region which is composed of highly basic and hydrophobic residues. This domain is essential for sequence specific DNA binding. The two regions are discussed in detail below. It is important to note that the sequence of the leucine repeats as well as that of the basic region, is highly conserved within the fos and jun families [12].

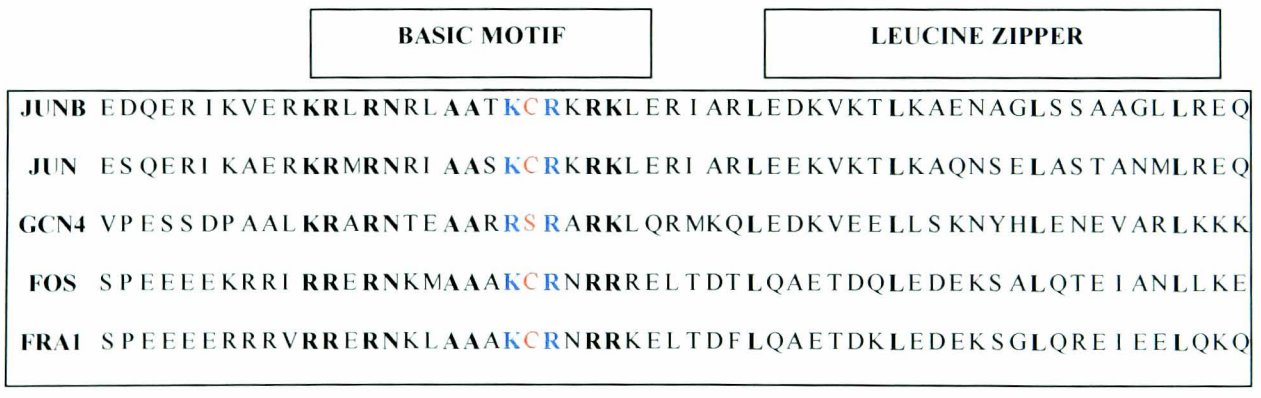


Figure 2 The 'Basic motif' and 'Leucine repeat' of the Fos, Jun and GCN4 Proteins **[19]**

1.3.1 DNA Binding of Fos-Jun Heterodimers

Although no direct structural information or data regarding the interaction of the binding domain of the AP-1 proteins with DNA is available, two closely related models have been proposed. The structural features of these models, that is the 'induced helical fork' and the 'scissors grip' models are similar in principle [20-22]. In both cases the basic region-leucine zipper is shaped like a **Y**. The parallel leucine zipper region forms the stem of **Y** and the basic regions form α-helices that can be considered as the 'arms' of the **Y**. In both models the N-terminal region of the two-fold symmetric leucine zipper fits over the centre of the DNA binding site, and the helices from the basic region extend in opposite directions along the major grooves of the DNA, figure 3. The two models differ however in their predictions regarding the interaction of the helices of the basic domains with DNA. The α-helix from the basic region is believed to be straight, projecting away from the DNA after contacting three or four base pairs in the induced helical fork model [21], however the scissors grip model predicts that this helix is kinked, thus enabling it to follow the curve of the major groove [22].

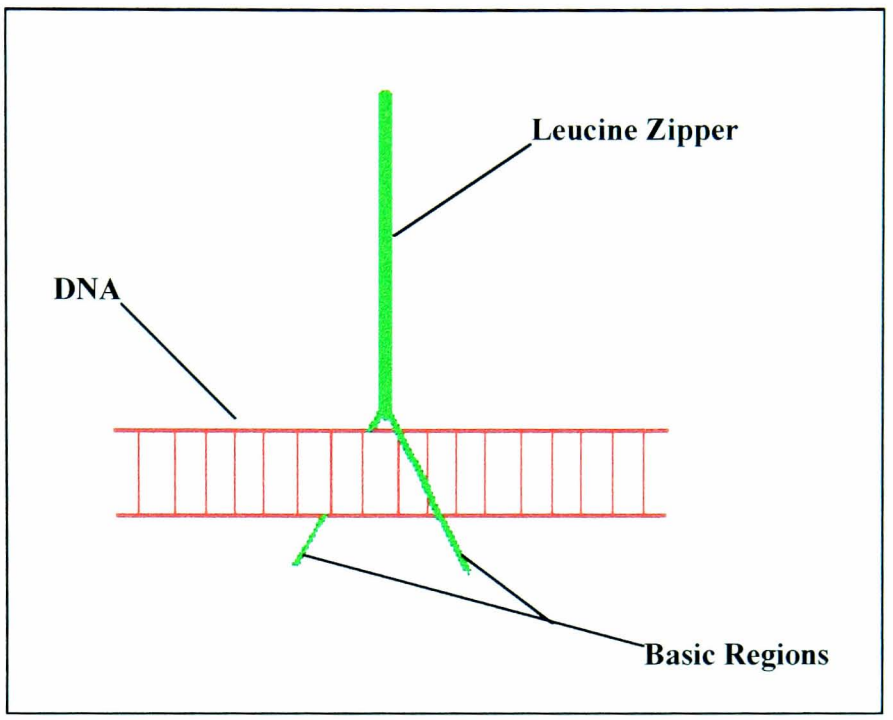


Figure 3 Schematic Representation of the DNA AP-1 Interaction

1.3.2 The Leucine Zipper

The leucine zipper is essential for the specific dimerisation between the fos and jun proteins. This domain is composed of two α-helices that are approximately 30 amino acids long and are characterised by a heptad repeat of Leu residues [23]. These α-helices interact in a parallel orientation to form a structure reminiscent of a coiled coil [22]. The side-chains of the Leu residues from one α-helix interdigitate with those from a second, thus forming strong hydrophobic interactions that mediate dimerisation [24]. It seems that this parallel interaction of the dimerisation (or leucine zipper) domains serve to juxtapose the basic regions (binding domains) of the fos and jun proteins, each of which is therefore

able to contact both strands of the AP-1 binding site [25]. Only as the dimer is the strong and sequence selective binding of the AP-1 protein achieved [25].

Jun proteins by themselves can bind as homodimers to the AP-1 and CRE enhancer elements in DNA, fos alone however, is unable to dimerise under physiological conditions and hence incapable of binding either site [26]. The heterodimeric protein i.e. fos-jun binds the DNA AP-1 and CRE sites with a much greater affinity than the jun homodimers. This observation may either be explained by the formation of more stable fos-jun dimers compared with the jun-jun dimers or by the high density of basic residues found in the fos component which makes the heterodimer more electrostatically compatible with the backbone of DNA [27] which is negatively charged.

1.3.3 The Basic Region

The basic region consists of a 16 amino acid residue consensus which is required for sequence-specific DNA binding by the AP-1 transcription factor [28]. This domain is rich in arginines (Arg (K)) and lysines (Lys (L)), but also contains other residues that are conserved throughout the bZip proteins. This region binds to non-specific DNA sequences in a partially helical conformation thus allowing the protein to effectively 'search' the DNA for its recognition sequence i.e. the AP-1 site. Once this sequence has been recognised the basic region becomes completely helical and forms a number of H-bonds and van der Waals contacts with the nucleotide bases found in the major groove of DNA.

1.3.4 AP-1 Activation

DNA binding of the AP-1 is actually modulated by the redox state of a single conserved Cys residue found in the basic DNA binding domains of the fos and jun proteins [29]. This Cys residue, which is mutated to serine (Ser (S)) in the oncogenic homologs of c-fos and c-jun, i.e. v-fos and v-jun respectively, is flanked by basic amino acids and is located in a highly conserved triamino acid sequence i.e. Lys-Cys-Arg (KCR). This particular sequence is found among all of the fos and jun related proteins and in several of the ATF/CREB proteins [11], figure 4. A similar motif is also present in the DNA binding domains of the Myb (KQCR) and NF-κB (KICR) proteins, both of which bind DNA in a redox dependent manner [30].

The reactive nature of the Cys residue which is enhanced by the local basic environment makes it readily susceptible to oxidation and thus an attractive candidate for regulation by a redox mechanism [29]. Thiol reduction of this critical Cys residue either by chemical reducing agents or nuclear extracts (e.g. redox factor nuclear protein) generates a free sulfhydryl group which permits DNA binding of the AP-1 transcription factor and hence activates transcription and cell growth [11]. Oxidation of this residue, however renders the AP-1 inactive by converting the Cys residue to a state that is not permissive for DNA binding and thus prevents transcription. Under such conditions the Cys residue is transformed to either one of two reversible products i.e. a sulfenic (RSOH) or a sulfinic (RSO_2H) acid, as opposed to the irreversibly oxidised form sulphonic acid

acid (RSO_3H) and the more common reversible disulphide oxidation product [29]. Substitution of this critical Cys residue in the DNA binding domains of the fos and jun proteins with Ser, however results in increased DNA binding activity and loss of redox control [5].

Fos	R	R	E	R	N	K	M	A	A	A	K	**C**	**R**	N	R	R	R	E	L	T
FosB	R	R	E	R	N	K	L	A	A	A	K	**C**	R	N	R	R	R	E	L	T
Fra1	R	R	E	R	N	K	L	A	A	A	K	**C**	R	N	R	R	K	E	L	T
Fra2	R	R	E	R	N	K	L	A	A	A	K	**C**	R	N	R	R	R	E	L	T
Jun	K	R	M	R	N	R	I	A	A	S	K	**C**	R	K	R	K	L	E	R	I
JunB	K	R	L	R	N	R	L	A	A	T	K	**C**	R	K	R	K	L	E	R	I
JunD	K	R	L	R	N	R	I	A	A	S	K	**C**	R	K	R	K	L	E	R	I
CRE	R	L	M	K	N	R	E	A	A	R	E	**C**	R	R	K	K	K	E	Y	V
B	R	L	M	K	N	R	E	-	A	R	E	**C**	R	R	K	K	K	E	Y	V
ATF1	F	L	E	R	N	R	A	A	A	S	R	**C**	R	Q	K	R	K	V	W	V
ATF2	R	R	E	R	N	K	I	A	A	A	K	**C**	R	N	K	K	K	E	K	T
ATF3																				

Figure 4 Sequence Alignment of the Basic Motifs of the Fos, Jun and ATF Proteins [11]

The exact mechanism responsible for redox control of DNA binding activity of the AP-1 transcription factor is not yet clearly understood. However a stimulatory factor present in mammalian nuclear extracts has been shown to stimulate the binding of the fos and jun proteins. This bifunctional protein, designated redox factor (Ref-1), in the absence of sulphydryl reducing agents maintains the reduced state of the Cys residue required for DNA binding of the AP-1 protein [5,29]. Ref-1 has also been shown to possess an endonuclease activity involved in the repair of DNA [11,29]. This protein enhances the affinity of the fos and jun

proteins for DNA indirectly and does not participate in the DNA-protein interaction [29]. Interestingly, Ref-1 itself is subject to redox control and its activity is augmented by thioredoxin, thus suggesting that a redox cascade could be involved in AP-1 regulation [11,25].

1.4 AP-1 Inhibition

The implication of various members of the bZip family of eukaryotic transcription factors and in particular the AP-1 in the induction of certain types of cancer has aroused a great deal of interest in these proteins. Although the function of these proteins in tumour growth is well documented no structural data on the interaction of the AP-1 with its cognate DNA sequence is yet available. However the solution structure of the yeast transcription factor GCN4 which shares a homologous DNA binding domain to the fos and jun proteins has been resolved and is therefore used in the study of the AP-1 and its interactions with DNA. The crystal structure of GCN4 complexed with the AP-1 oligonucleotide binding sequence has also been resolved [17], and is better described by the induced helical fork model (figure 5).

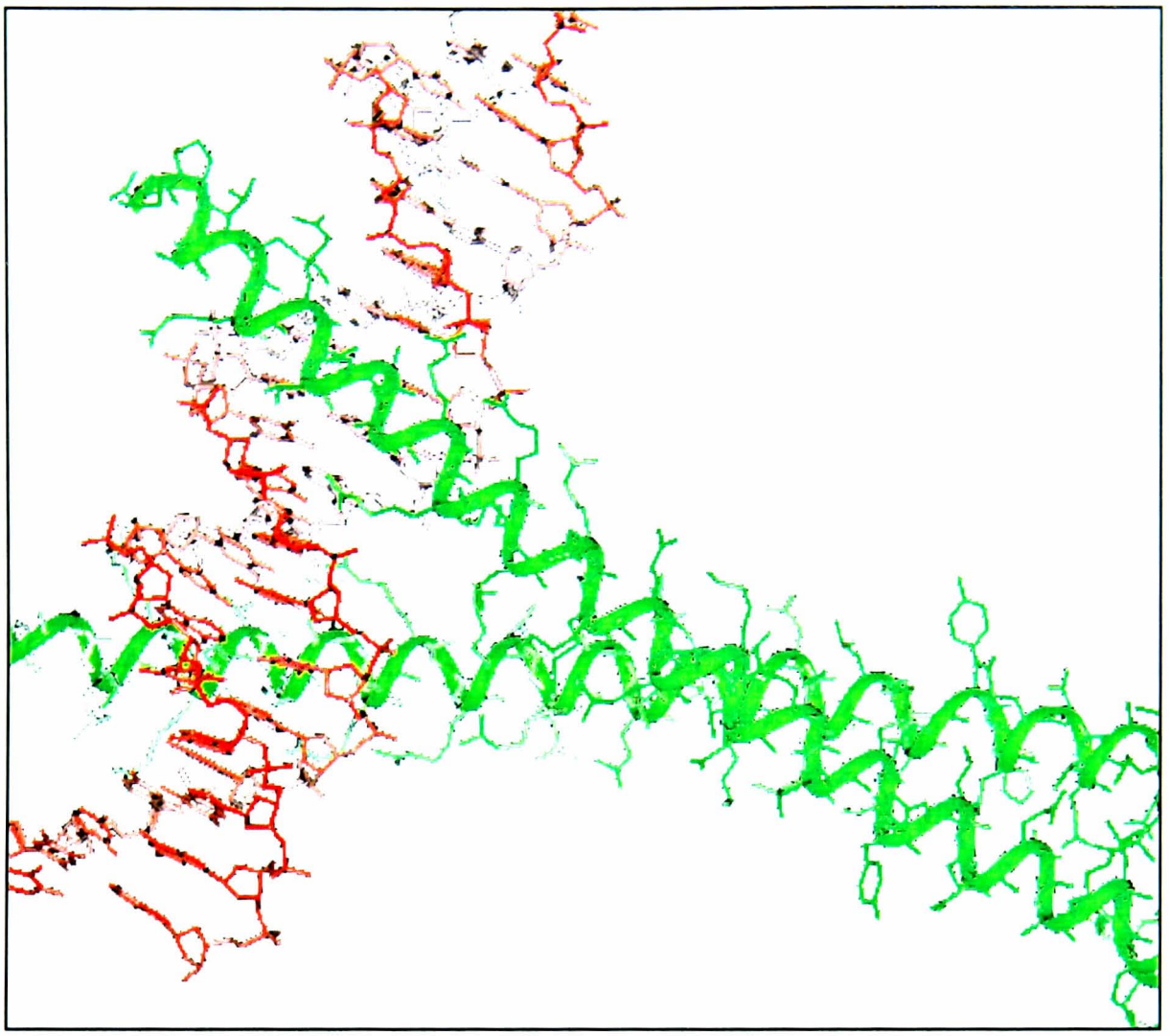


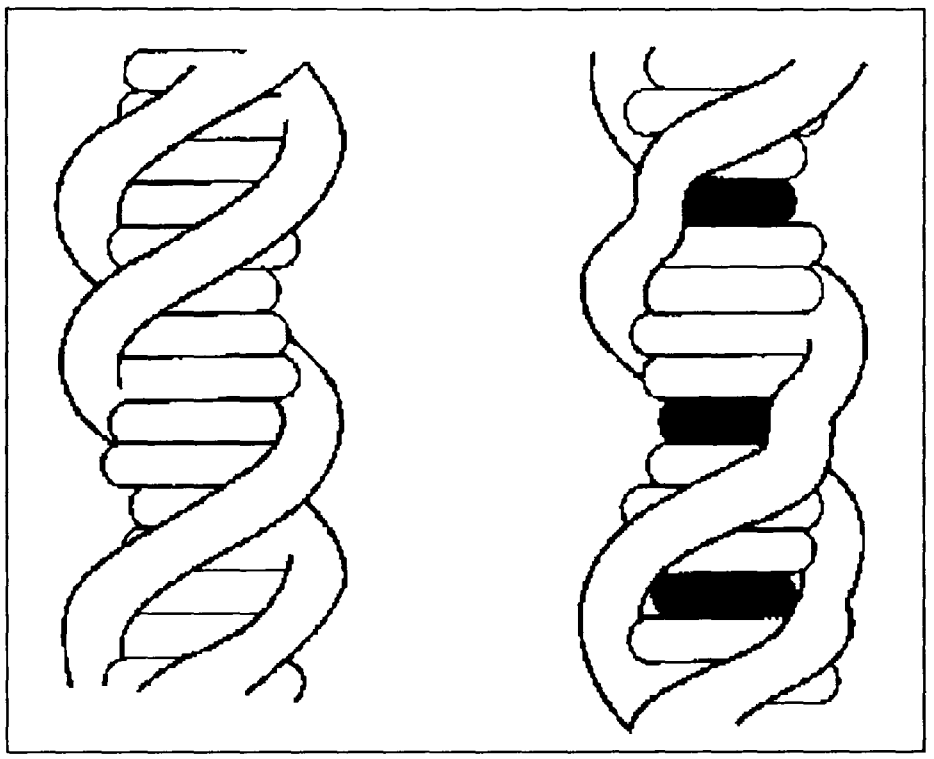
Figure 5 A Computer Model of GCN4 Complexed with the DNA AP-1 Consensus Sequence [17]

The redox control of the AP-1 transcription factor plays an important role in both cell proliferation and malignant transformation, the latter of which is more likely to occur under conditions of oxidative stress where the tumour cells have very little or no oxygen [13]. The low level of oxygen in these tumour cells thus maintains the Cys residue of the AP-1 in a reduced state which permits DNA binding of the protein and hence activates transcription and tumour growth. It is possible to conceive three general methods of inhibiting the AP-1, firstly to prevent dimerisation of the fos and jun proteins, secondly to provide an agent that binds to the protein dimer and hence prevents it from binding to DNA and thirdly to design a drug that binds the DNA AP-1 site and thus prevents DNA binding of the protein. Since DNA is arguably the best characterised target in cancer therapy, with a number of clinical agents already established with strong DNA binding moieties, it is natural to consider an approach based on method three in the first instance, preventing the AP-1 protein from activating transcription. The loss of transcription of important genes responsible for tumour cell survival and division would provide a novel and selective route for the treatment of cancer.

1.5 DNA Binding Agents

1.5.1 Intercalation

The process of intercalation entails the insertion of a flat aromatic chromophore between the base pairs of DNA [31-34]. In order to accommodate the intercalating molecule, the duplex unwinds in the region of intercalation, thus increasing the separation between the planes of the base pairs (figure 6). This results in both the lengthening and distorting of the double helix which affects its interaction with DNA associated enzymes and nuclear proteins [33]. Intercalation therefore interferes with the role of DNA as an effective template for DNA and RNA synthesis i.e. replication and transcription [32].



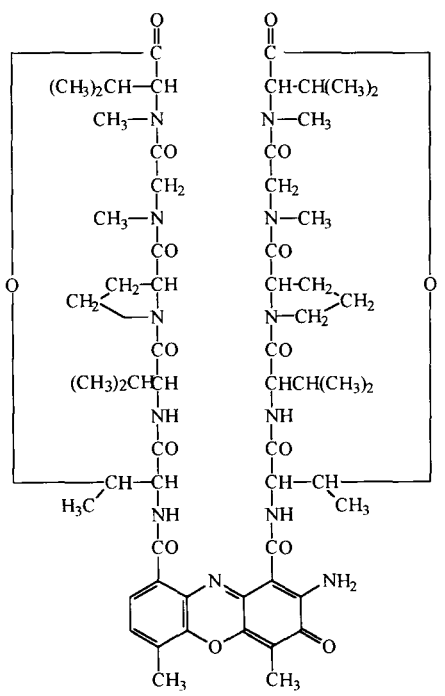
(a) *(b)*

Figure 6 (a) Native DNA (b) Intercalated DNA [34]

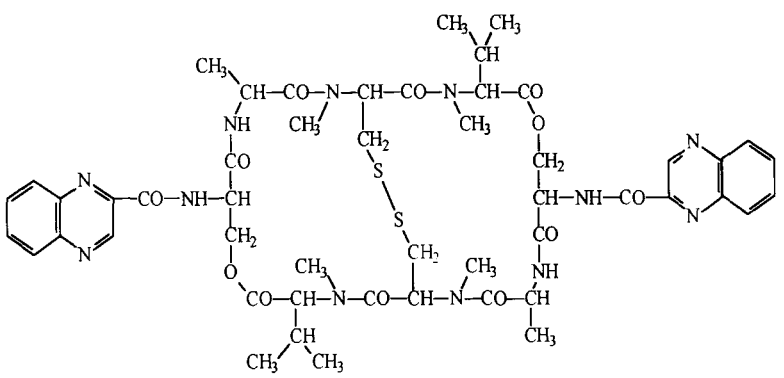
1.5.2 Natural Hybrid Peptides

The naturally occurring peptide antibiotics such as the actinomycins and echinomycins (figure 7) are potent cytotoxic agents. These anti-tumour antibiotics, which are selective inhibitors of DNA directed RNA synthesis, are characterised by chromophoric intercalating and bis-intercalating groups attached to peptidic moieties [35]. The former, i.e. the intercalating group, facilitates DNA binding by the mode of intercalation, where as the latter provides nucleotide sequence specificity. Considering the effect of the intercalating chromophore on the DNA binding of nuclear proteins, many intercalating agents such as the anti-

tumour antibiotics, actinomycin and echinomycin can be thought of as naturally occurring transcription factor inhibitors.



Actinomycin



Echinomycin

Figure 7 Natural Occurring Anti-tumour Antibiotics

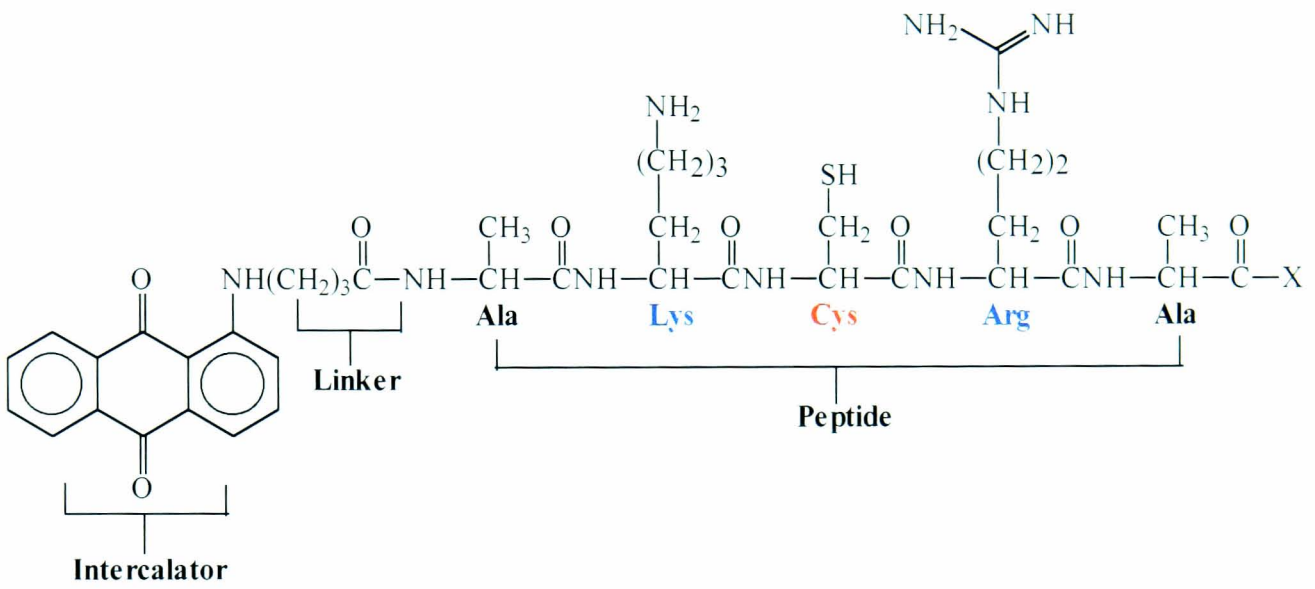
1.6 Aims of this Study

The aim of this project is to design, synthesise and analyse a number of potential redox sensitive transcription factor inhibitors. These should be able to compete with AP-1 for its cognate DNA sequence. The inhibitor designed must therefore be specific for the AP-1 DNA binding site, redox active and selective under oxidative stress or hypoxic conditions.

The design principle of the naturally occurring peptide antibiotics has been adopted as a paradigm for the potential AP-1 transcription factor inhibitors. It is anticipated that the specificity of these drugs for the AP-1 site can be achieved by designing peptides that mimic the DNA binding domains of the AP-1 transcription factor. A redox switch will ensure that the inhibitors are selective under oxidative stress/hypoxic conditions and the incorporation of an intercalating moiety will facilitate initial DNA binding and hence nuclear accumulation of these agents.

1.6.1 A Potential Hypoxia-Selective Anti-Tumour Agent

Taking into consideration the information obtained from previous studies and the factors necessary for the binding of the fos-jun heterodimer to the DNA AP-1 binding site, the structure shown in figure 8 has been proposed as an example of a potential AP-1 transcription factor inhibitor.



X = OH or NH_2

Figure 8 A Potential AP-1 Transcription Factor Inhibitor

The specificity of this anti-tumour agent will be provided by the peptide moiety, the sequence of which has been based on the factors necessary for the binding of the fos-jun heterodimer to the DNA AP-1 site. It is anticipated that DNA binding of this agent will compete with the binding of the AP-1 transcription factor, and therefore prevent transcription and tumour growth. The overall objectives of this project have been summarised below:

- Synthesis of the proposed structure (figure 8) and its analogues using various peptidic sequences (principally bearing the KCR motif), linker groups and chromophores.

- Attempt the synthesis of an α-helical constrained peptide bearing the KCR motif and the determination of its effect on the DNA binding of the drug.

- Biophysical evaluation of the peptides and peptide conjugates to assess relative binding affinities to DNA and selective inhibition of AP-1 protein binding to DNA.

- Structural evaluation of the drugs synthesised using Circular Dichroism (CD), 2D-Nuclear Magnetic Resonance (NMR) and molecular modelling techniques to establish the secondary structure adopted by the peptides and their respective conjugates.

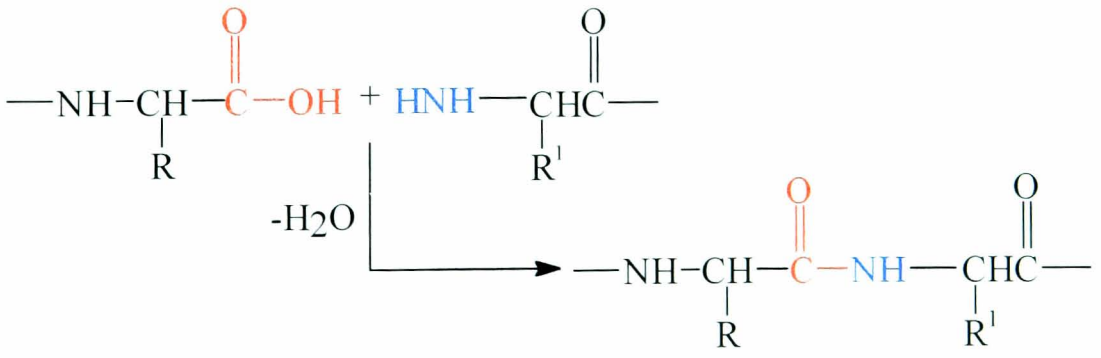
- Conduct theoretical studies on the peptide/DNA complexes using computerised molecular modelling techniques.

2 TECHNIQUES

The following chapter provides a background to the techniques employed in peptide synthesis and to the methods used to evaluate, both biologically and structurally, the transcription factor inhibitors synthesised in this study. The results obtained from the synthesis and evaluation of the inhibitors are presented and discussed in chapters 3 and 4 respectively.

2.1 Peptide Synthesis - An Overview

Peptide synthesis involves a series of protection, deprotection and coupling reactions. The coupling or amide bond forming reaction essentially involves the condensation of the two amino acid residues with the elimination of water (figure 9).

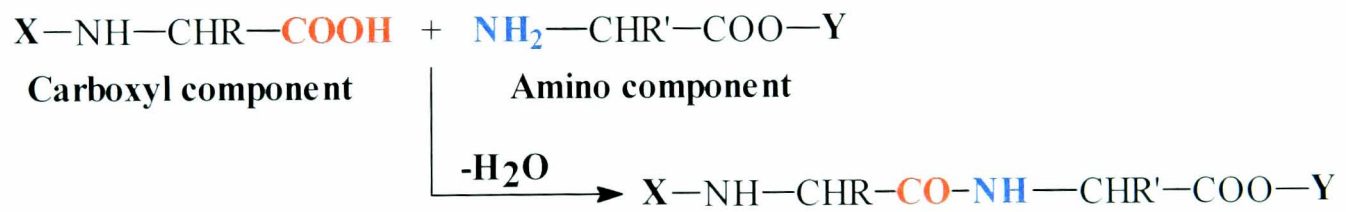


R and R^1 = Amino acid side-chains

Figure 9 Peptide Bond Formation

2.1.1 Protection

α-Amino acids, although chemically varied, all contain an amino group, carboxyl group and a distinctive R (side-chain) group with the exception of glycine, all of which are attached to the single α-carbon atom [36]. Both the amino and carboxyl groups are reactive centres that behave as nucleophilic and electrophilic entities respectively. Thus in order to prevent polycondensations, i.e. the formation of higher polymers, it is necessary to block those functional groups that are not required to participate in the peptide bond forming reaction [37]. That is to say that protecting groups are required for the amino function of the amino acid to be activated (i.e. the carboxyl component) and the carboxyl group of the amino acid to be acylated (i.e. the amino component),figure 10.



X and **Y** are protecting groups of the α- amino and carboxyl groups respectively.

Figure 10 Amino Acid Protection

These protecting groups not only suppress the reactivity of the functional groups but also destroy the zwitterionic character of amino acids when in solution, in which the amino and carboxyl groups become protonated and deprotonated

respectively. It is essential to prevent the formation of zwitterions (figure 11) as this diminishes the solubility and reactivity of the residue.

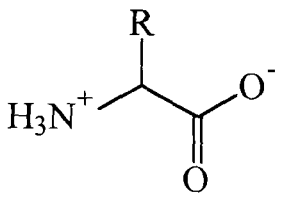


Figure 11 The Internal Salt or Zwitterion

Elongation of the dipeptide can be achieved by simply removing one of the protecting groups, conventionally the amino protecting group and then coupling with a carboxyl-activated amino acid [38]. It is essential that the protecting group of one functionality i.e. the amino group, can be selectively removed under conditions that do not effect the masking of the other (carboxyl function) and *vice versa*. It is also important that the peptide bond is not cleaved during the process of deprotection. Protection of the functional groups present in the side-chains of many amino acid residues, such as Arg and Lys, is also necessary as these groups can also participate in the peptide bond forming reaction. These groups require orthogonal protection for at least one of the α-functions, which means therefore that selective deprotection can be achieved [37]. For instance the α- and ε-amino groups of Lys may be masked with acid (e.g. t-butyloxycarbonyl group (Boc)) and base (e.g. 9-fluorenylmethoxycarbonyl group (Fmoc)) labile protecting groups respectively. This essentially means that the α-amino function can be selectively

deprotected without effecting the protecting group used to mask the ε-amino group and *vice versa*, see figure 12.

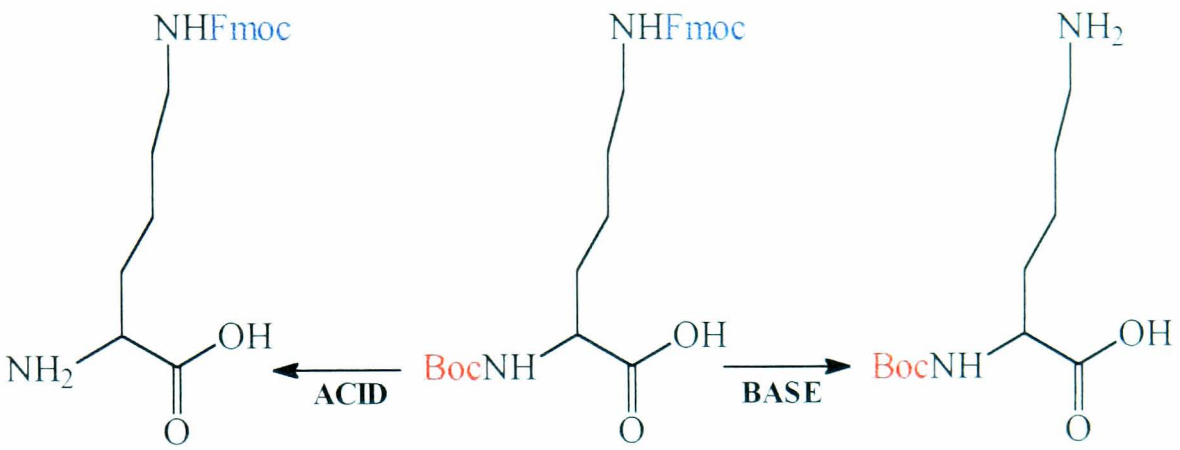


Figure 12 Orthogonal Protection of the Lys Residue

2.1.2 Amino and Carboxyl Protecting Groups

Protecting groups employed to mask the amino function of a residue are required to suppress both the basic nature and nucleophilic reactivity of the nitrogen atom. This is achieved by either draining its electron density away into an appropriate substituent (i.e. delocalisation of the lone pair of electrons) or by concealing it behind a screen of steric hindrance [38]. Protection of the amino group can be accomplished by simply transforming it to its corresponding amide. However, although this method of protection renders the amino function inert, the resultant amidic bond to the protecting group may not be readily distinguished from the amidic backbone of the peptide. Under these circumstances, deprotection may result in peptide cleavage. Furthermore, the electron withdrawing nature of the

amide group enhances the acidity of the α-proton and therefore results in racemisation.

The protecting groups more commonly employed to mask the amino function are carbamates. They are related to the amide group and include the Boc and Fmoc groups [37,39]. These particular masking groups are more desirable than the amides as the carbonyl groups are less electron withdrawing. This is due to the delocalisation of a lone pair of electrons from the oxygen into the carbonyl group, lowering the electron demand on the protected nitrogen. Additionally, this electron donation by the oxygen atom renders the carbamate carbonyl less susceptible to nucleophilic attack [37]. The final advantage of carbamate protecting groups is that they can be deprotected under conditions that relate to the hydrolysis of the ester component of the $ROCONHR^1$ moiety. Subsequent spontaneous decomposition of the resultant carbamic acid reveals the free amino group.

Although the carboxyl function of an amino acid residue is less nucleophilic than the amino group with respect to acylation, it still requires protection. This not only aids solubility of the peptide in organic solvents but also allows unambiguous activation in the presence of a coupling reagent (i.e. only the unprotected carboxyl group is activated). The usual means of carboxyl protection is esterification, the most common groups being the alkyl esters such as the methyl and ethyl esters. Esters are also commonly employed in solid phase

peptide synthesis which often involves the attachment of the C-terminal amino acid residue to the resin through an ester linkage, thus providing both an attachment point and C-terminal protection. Typically such esters are substituted benzyl esters which are cleaved by acidolysis to yield the desired peptide terminus. In the context of this work, the side chains of Arg, Cys, Lys and Ser all required protection, the protecting groups to be employed in this synthesis are described in table 1.

Table 1 Amino Acid Protecting Groups

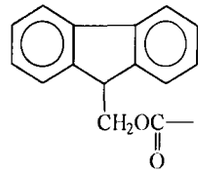
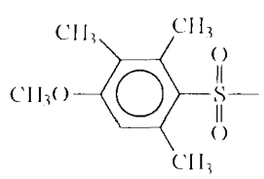
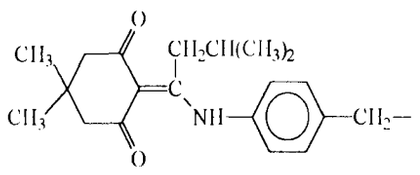
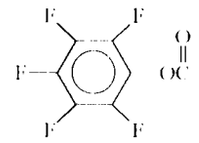
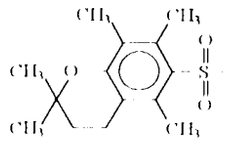
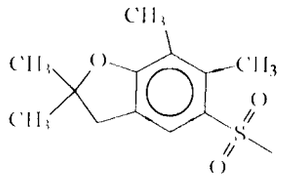
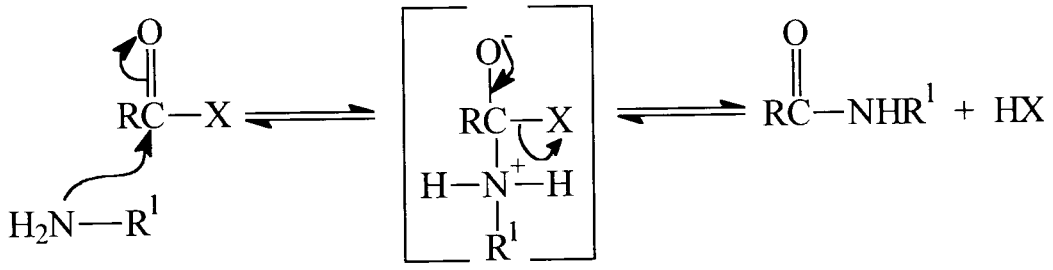
Protecting Group	Abbreviation	Structure	Function Protected	Deprotection Conditions
t-Butyl	t-Bu	$(CH_3)_3C$—	OH	TFA
t-Butyloxycarbonyl	Boc	$(CH_3)_3C$—O—CO—	NH_2	TFA/HCl
Fluorenylmethoxycarbonyl	Fmoc		NH_2	20% Piperidine
4-Methoxy-2,3,6-trimethyl-benzenesulphonyl	Mtr		NH_2	TFA
4-{N-[1-(4,4-Dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]amino}benzyl	ODmab		CO_2H	2% Hydrazine

Table 1 (Continued) Amino Acid Protecting Groups

Protecting Group	Abbreviation	Structure	Function Protected	Deprotection Conditions
Methyl Ester	**OMe**	—OCH_3	**CO_2H**	**ROH/OH^-**
Pentafluorophenyl Ester	**Opfp**		**CO_2H**	**1% TFA**
2,2,5,7,8-Pentamethyl-chroman-6-sulphonyl	**Pmc**		**NH_2**	**TFA**
2,2,4,6,7-Pentamethyldihydrobenzo-furan-5-sulfonyl	**Pbf**		**NH_2**	**TFA**
Trityl	**Trt**	$(Ph)_3C$—	**SH**	**Mild Acid**

2.1.3 Activation

In order to convert the carboxyl group of an amino acid into an acylating agent. the OH group must be replaced with, or activated by an electron withdrawing group [38]. This is required to increase the electrophilicity of the carbon of the carboxyl group, and provide an improved leaving group, hence greatly facilitating nucleophilic attack by the nitrogen of the amino component. Furthermore, as shown in the reaction mechanism in figure 13, the electron-withdrawing group should ideally facilitate deprotonation of the quaternised nitrogen. This favours the production of the product from the intermediate over decomposition back to the starting materials.

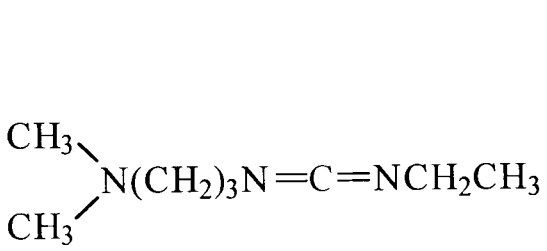


X = Activating group; R, R^1 = Amino acid side-chains

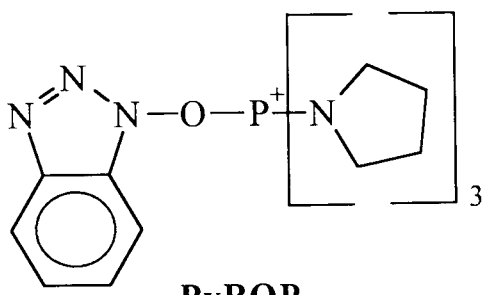
Figure 13 Carboxyl Activation

Activation can be a two step process i.e. initial carboxyl over-activation which is then followed by substitution with a nucleophile that provides a secondary activating function of lower reactivity [38]. Two of the most commonly employed coupling agents in peptide synthesis are 1-ethyl-3-(3-dimethylamino-

propyl)carbodiimide (EDC) and benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) (figure 14), the former of which is often used in solution phase synthesis and the latter in solid phase peptide synthesis (SPPS). The use of coupling agents are advantageous as they can be added to a mixture of the carboxyl and amino components, thus allowing activation and coupling to proceed concurrently. These coupling agents promote amide bond formation by reacting with the carboxyl group of the amino acid and activating it towards nucleophilic substitution.



EDC

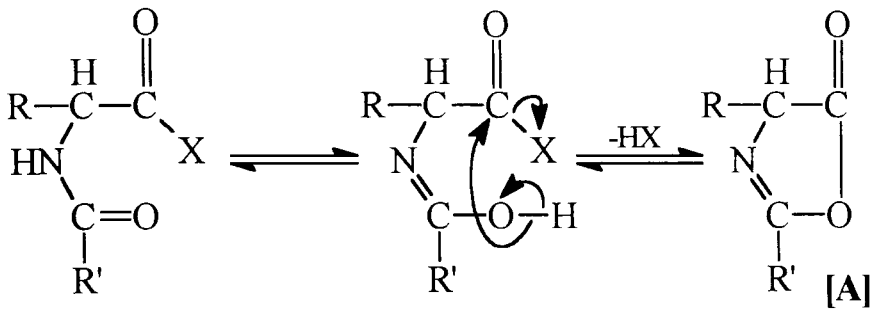


PyBOP

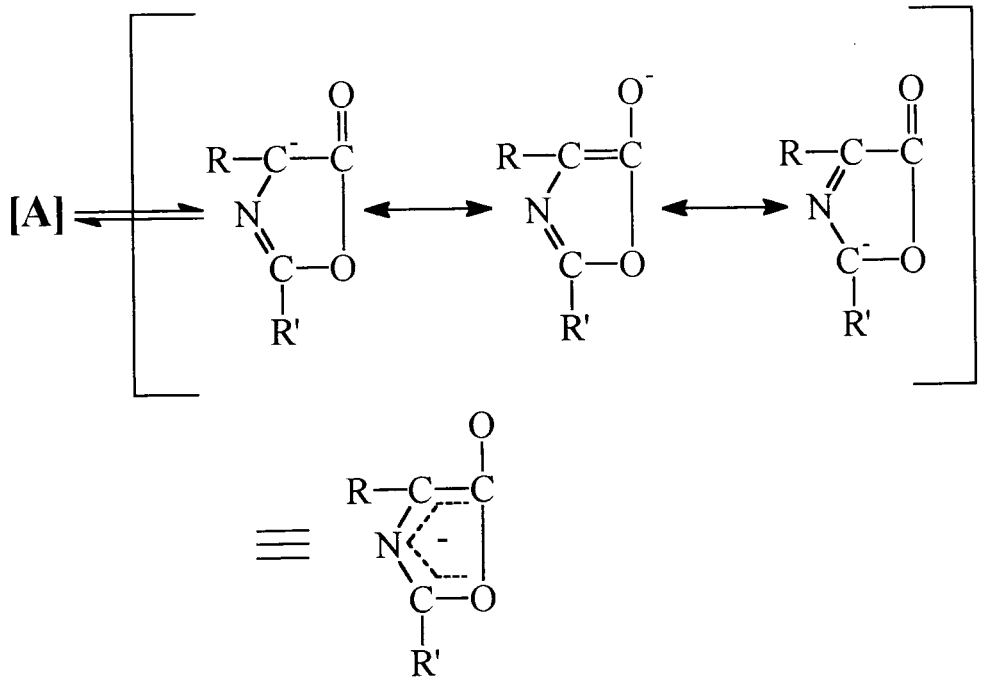
Figure 14 Coupling Agents, EDC and PyBOP

All amino acids (except for glycine) are optically active and hence prone to racemisation during the coupling process. Increased electrophilicity of the carbonyl carbon by coupling agents leads to an increase in the acidic nature of the α-hydrogen thus increasing the possibility of racemisation [37,38], the most important mechanism of which involves the formation of activated acylamino acids as shown below:

Racemization



Racemization of azlactone [A] results from the abstraction of the acidic proton from the chiral centre, the ease of which is due to the resonance stabilisation of the carbanion generated:



In order to prevent racemization an auxiliary nucleophile such as N-hydroxybenzotriazole (HOBt) is required. HOBt (figure 15) is termed an auxiliary nucleophile as it destroys the highly activated intermediates and thus remits proton abstraction from the chiral carbon atom preserving chiral purity

[37,38]. The addition of such a nucleophile is prerequisite in reactions in which carbodiimides such as EDC are employed, however where PyBOP is used, such a reagent is not required as HOBt is generated in situ, see section 3.2.3.

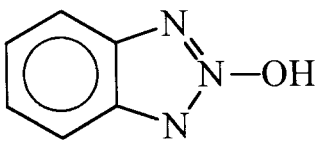


Figure 15 N-Hydroxybenzotriazole

2.1.4 Solution Phase Peptide Synthesis

Whilst useful for the preparation of large quantities of short peptides, solution phase synthesis is not without inherent difficulties. It employs techniques of solvent extraction, filtration, evaporation, recrystallisation and possibly chromatography, all of which are both time consuming and lead to loss of product. The total number of steps involved in the synthesis of even moderately sized peptides can therefore be quite substantial. Furthermore, purification by recrystallisation and chromatography become increasingly difficult as the peptide chain length increases. The problems encountered with peptide purification and the low yields make this a less favourable method of peptide synthesis.

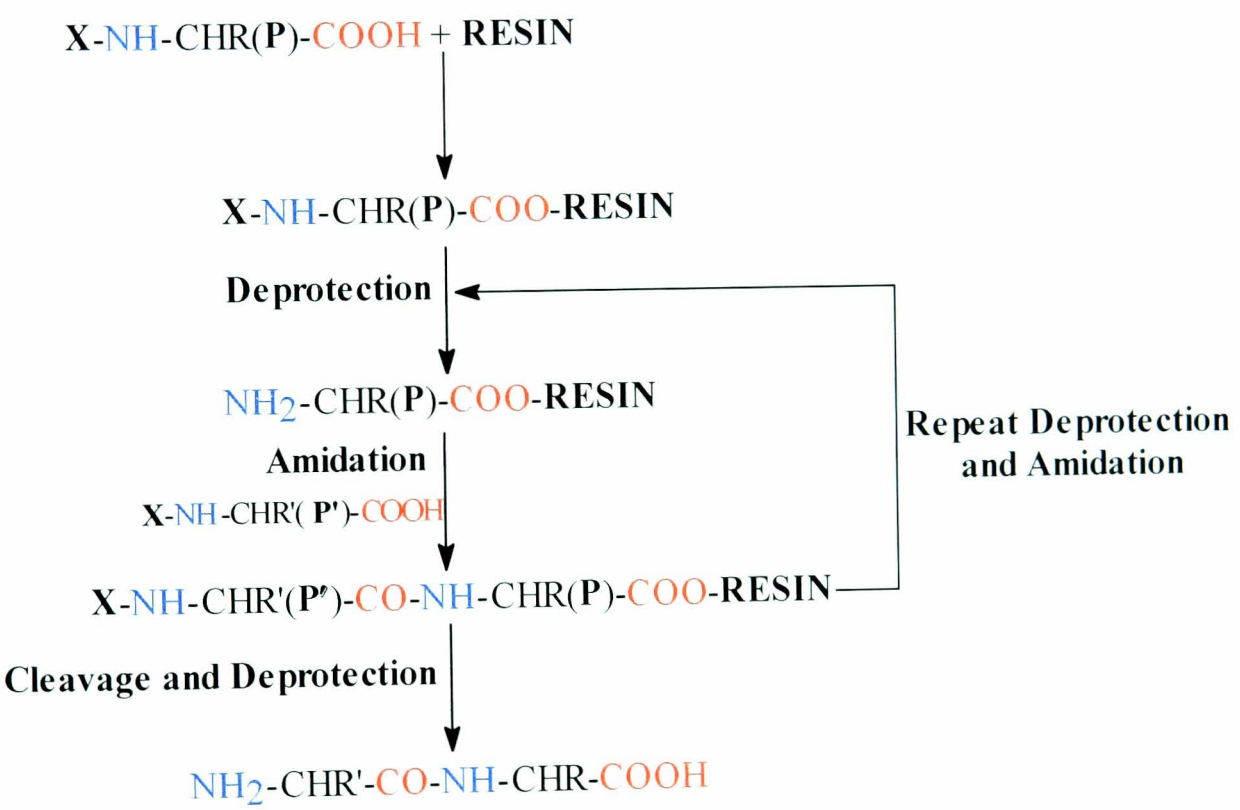
2.1.5 Solid Phase Peptide Synthesis

The fundamental premise of Solid Phase Peptide Synthesis (SPPS) is the sequential addition of N-α protected amino acid residues to a peptide linked to an insoluble polymeric support [40]. The 'growing' peptide sequence remains attached to the support or resin via a linker, through its C-terminus throughout the synthesis, allowing unreacted reagents and by-products to be removed by simple washing procedures.

There are two techniques employed in SPPS, namely, 'Batchwise' and 'Continuous flow' synthesis. In the former technique the synthesis is carried out in a filter reaction vessel, which allows the support to be filtered and resolvated at the end of each stage. However, in the latter, the resin remains solvated throughout the synthesis and is contained in a column through which the reagents and solvents are pumped continuously [41]. Generally only the Fmoc strategy is used with the continuous flow method, however both Fmoc and Boc chemistry can be used in batch synthesis. In both cases, the reagents can be added and removed under manual or computer control.

SPPS essentially involves the reversible attachment of the first amino acid residue to a solid support, which is then followed by a repeated cycle which involves deprotection (i.e. liberation of the free amino group) and acylation with the next N-α protected residue. The general scheme of SPPS is shown in figure 16. The resin and its attached peptide is washed thoroughly at the end of each step with an

appropriate solvent, this removes any by-products formed and any unreacted reagents remaining in the reaction vessel. This cycle is repeated until the desired peptide sequence is achieved. The product is then isolated by treating the support with a strong acid such as hydrogen fluoride or trifluoroacetic acid (TFA), which allows the simultaneous cleavage of the peptide from the resin and the removal of any acid-labile protecting groups present in the side-chains of the amino acid residues [41].



X= N-α protecting group; P and P^1 = Side chain protecting groups

Figure 16 A General SPPS Protocol

SPPS not only avoids the purification of peptide intermediates but it also minimises the physical loss of the peptide product, as it is retained in the same vessel throughout the synthesis. The relative ease with which soluble reagents are removed is also advantageous as it allows an excess of the reagents to be used, and therefore facilitates a high product yield.

The main disadvantage associated with SPPS is that the peptide intermediates cannot be purified and that possibilities for their characterisation are limited. The analytical methods often employed in solution phase peptide synthesis are not always practical in SPPS, for example spectroscopic study is often impeded by the presence of the polymeric resin support [41]. This can be rather problematic in the detection of truncated sequences or prematurely terminated chains i.e. deficient peptides go undetected and therefore create further problems in the purification of the final product. The formation of such undesirable products however, can be avoided by monitoring the reactions to establish the completeness of acylation and to ensure that deprotection of the α-amino group is complete. The former can be achieved by performing a Kaiser test [42] which determines the presence of unreacted primary amino groups, therefore indicating the completion of acylation. Deprotection, however, can be monitored by employing an in-line Ultra Violet (UV) spectrophotometer which detects the cleaved Fmoc derivative dibenzofulvene (see section 3.2.4.1), this however is only possible in continuous flow synthesis.

Another major problem associated with SPPS is peptide-chain aggregation, which is due to either hydrophobic interactions or interchain hydrogen bonding. This typically occurs between 5-15 residues from the C-terminus and can lead to incomplete coupling and deprotection [40,43]. However, peptide chain aggregation can be avoided by the addition of soluble ureas to the acylation reaction, which disrupt hydrogen bonding and can lead to increased coupling yields. Despite the problems associated with SPPS, this method is still preferred and hence commonly employed.

2.1.6 Protein Structure

Many of the bonds in a polypeptide/protein chain allow free rotation of the atoms giving the peptide backbone great flexibility. This therefore means that the polypeptide is able to apparently adopt an almost unlimited number of conformations, however this is dependent on its primary structure [44]. The conformation anticipated in the peptides employed in this study is the alpha helix. This secondary structural motif (figure 17) is generated when the backbone turns regularly about itself to make a rigid cylinder in which each residues' carbonyl group forms a hydrogen bond with the amide NH group of the residue four amino acids along the chain.

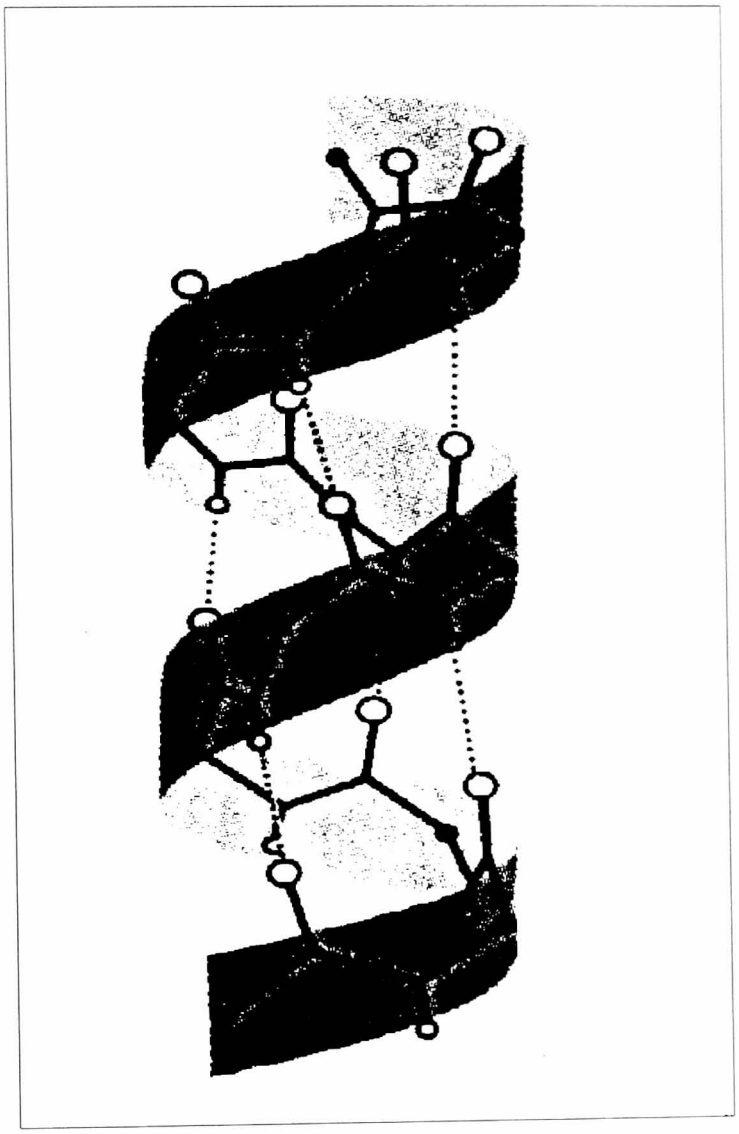
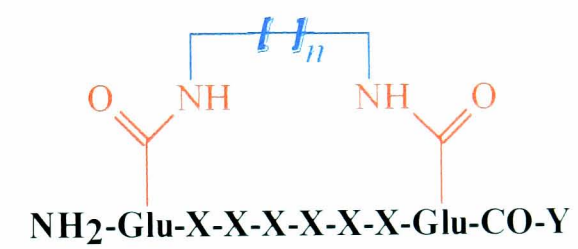


Figure 17 An Alpha Helix [44]

Investigations of the chemical and physical properties of isolated helices have long been hampered by the inability of most linear peptides to sustain an α-helical conformation in aqueous solution. It is because of this that several methods for constraining short peptides to an α-helical conformation have been developed. Such approaches or methods have involved the incorporation of salt bridges [45], modification of the solvent [46] and more favourably the formation of disulphide [47,48] and amide bridges [49,50]. The method to be adopted in this synthesis is

one described by Phelan and co-workers [50] in which glutamic acid residues at positions *i* and *i*+7 are bridged together with an alkanediamine chain, figure 18. In the first instance attempts at constraining only a single peptide were made using this method as at this stage it was not known whether or not the alkanediamine bridge would mask the binding moieties of the peptide and hence prevent/hinder its interaction with the DNA AP-1 site. Further details of this approach will be discussed in section 3.7.



n = Number of methylene groups; X = Amino acid residues; Y = NH or OH

Figure 18 Constrained Peptide

2.2 Biophysical Studies

Spectroscopic studies, together with electrophoretic mobility shift assays (EMSA) were used to ascertain information regarding the biological activity of DNA binding ligands. The former can be used to determine the mode of binding and affinity of a drug for DNA, whereas the latter is employed to establish the selectivity and specificity of a drug for a particular DNA sequence.

2.2.1 DNA Binding Studies Using Spectroscopic Methods

Plumbridge and Brown [51] were amongst the first to show that a combination of spectroscopic and fluorescence techniques can be used to positively identify the interactions of synthetic intercalators with nucleic acids. Both the nature of binding and the affinity of a drug for DNA can be determined using these methods. It is essential, however, that the techniques employed distinguish the intercalative mode of binding from other modes and also allow the determination of the affinity of the drug when intercalation is shown. Consequently the methods used in assessing DNA binding fall into two categories. In the first group of methods the results generated affords information on the nature of binding. The second group of methods, however, is of a quantitative nature and provides information on the binding affinity of a drug for DNA. The methods used can further be subdivided into those that monitor spectroscopic changes in the properties of the drug and secondly those that monitor changes in the physical properties of DNA.

2.2.2 Spectroscopic Studies

On binding of a drug to DNA there is a spectral change in the absorption characteristics and fluorescence spectrum. These spectroscopic methods are applicable to the examination of intercalating drugs since the λ_{max} value for intercalating drugs (~400nm) are at a longer wavelength than the absorption due to nucleic acids (260nm) [51,52]. Spectrophotometric and fluorescence methods

can therefore be employed to determine the nature of binding and the affinity of a drug for DNA.

Typically the absorption spectrum of a drug upon intercalating into DNA is shifted to a longer wavelength (bathochromic shift) and the maximum absorptivity is decreased (hypochromism) [53]. The combination of bathochromic shift and hypochromism make it likely that the spectra of free and bound drug will cross at least one point. Thus when the spectra of solutions containing a fixed concentration of drug and varying concentrations of DNA (from 0 to 10:1 of DNA to drug) are superimposed then all the spectra will pass through this point, which is termed an isosbestic point. This however will only occur if there is a single spectroscopically distinct bound form of the drug molecule in addition to the free drug. The appearance of an isosbestic point is therefore essential if such spectra are to be used to determine the affinity of the drug for DNA.

Consequently when an isosbestic point is observed the affinity constant (K) for the binding reaction and the number of sites per DNA phosphorous (n) can be determined by the method of spectrophotometric titration [32,51]. This method is based on the assumption that the interaction of a drug with DNA involves a reversible dynamic equilibrium, which can be represented as follows:

Drug + DNA $\rightleftharpoons$ Drug-DNA Complex

The concentration of the bound drug (c) can be related to the concentration of the unbound drug by the law of mass action:

$$K = r/c(n-r) \qquad \textbf{Equation 1}$$

Where K is the affinity constant and is the ratio of association and dissociation constants $K_1 + K_2$

r is the concentration of bound drug per DNAp (concentration of DNA is expressed in terms of phosphate)

c is the concentration of unbound drug and

n is the number of binding sites for drug per DNAp

Rearranging equation 1 to a linear form (equation 2) using the method of Scatchard [54] allows calculation of both K and n.

$$r/c = Kn - Kr \qquad \textbf{Equation 2}$$

A plot of r/c versus r should yield a straight line of gradient $-K$ and intercept on the r axis, which is equal to Kn.

2.2.3 Fluorescence Studies

Fluorescence spectroscopy, in much the same way as UV spectroscopy, can be used to characterise DNA-drug interactions [55]. It has previously been shown that the nature of interaction of drugs with DNA and the relative affinity of these drugs for DNA can be determined using fluorescence spectroscopy.

Ethidium bromide, a known intercalator [34,56] shows a large enhancement of quantum yield of fluorescence on binding to DNA and therefore is considered to be an ideal fluorescent probe. Hence if a compound binds to the same site in the DNA helix as the ethidium bromide it will compete with ethidium bromide for that site. Providing that the fluorescence of the ethidium bromide in the absence of DNA is not effected by the drug under investigation it is possible to examine the effect of that drug on the fluorescence enhancement of ethidium bromide when bound to DNA [57].

2.2.4 Thermal Denaturation Studies

The interaction of DNA binding drugs can also be studied by monitoring their effects on the physical properties of DNA. In particular viscometry [58,59] and thermal denaturation [58,59] of DNA-drug complexes can be used to provide further information about drugs that bind DNA. The latter technique was employed in this study to investigate the stabilisation of the DNA double helix to heat denaturation by the peptide conjugates. Thermal denaturation of DNA in the presence of drug leads to changes in its the physical properties. These changes

can be monitored where they are independent of the spectral properties of the drug [60].

Native DNA undergoes a transition to the denatured single stranded form when subjected to an increase in temperature. This transition results from the rupturing of the hydrogen bonds that hold the DNA strands together. Since the extinction coefficient of denatured DNA at 260nm is greater than that of native duplex DNA the rate of thermal denaturation can be followed spectrophotometrically as the temperature of the sample is increased. The temperature corresponding to half the increase in the relative absorbance is designated the melting temperature, T_m. The T_m is greatly influenced by the ionic strength of the buffer and the base composition of DNA [59], since, for example, a high GC content confers a higher thermal stability [59]. The presence of drugs that stabilise the DNA duplex through intercalation or other modes of binding (i.e. non-intercalative) will also lead to an increase in T_m of DNA [58,59], since more energy is required to separate the strands of the double helix. The increased stability imparted to the DNA helix by the drug can also be used as an indicator of its binding affinity.

2.2.5 Electrophoretic Mobility Shift Assay (EMSA)

DNA binding proteins play a major role in gene expression through their involvement in transcription, replication and recombination. The study of DNA-protein interactions has been markedly facilitated in recent years by "gel retardation" or "gel mobility shift" assays [61,62]. This technique is based on the

observation that the electrophoretic mobility of a DNA fragment in gels is greatly retarded upon binding of a protein fragment [61]. These assays can therefore be employed to determine the selectivity, specificity and binding activity of DNA binding ligands.

2.3 Structural Studies

There are a number of techniques that can be used for determining the structure of peptides and proteins. NMR spectroscopy, Circular Dichroism (CD) and computerised molecular modelling techniques are amongst the most useful methodologies used in conformational analysis and DNA-drug interactions.

2.3.1 Nuclear Magnetic Resonance

The number of macromolecular structures that have been resolved using two-dimensional (2D) NMR techniques has dramatically increased over the past few years. Homonuclear correlated spectroscopy (COSY) and Nuclear Overhauser Enhancement Spectroscopy (NOESY) are the two most commonly employed 2D-techniques in the elucidation of the structure and function of biologically important molecules [63]. The spectra obtained from these experiments are presented in a two-dimensional manner (pseudo 3D) which allows much more information to be assembled and correlated than would be conceivable in a normal one-dimensional plot [64].

2.3.1.1 COSY Spectroscopy

COSY spectroscopy is often employed in disentangling complicated proton spectra as it allows the determination of how various protons in a molecule are coupled to each other i.e. it establishes proton-proton coupling. Since coupled protons are usually separated by two or three bonds, the connectivity and very often the chemical structure of a molecule can be derived from a COSY spectrum [63]. This technique is therefore extremely important in the structural analysis of complex organic and biochemical compounds, which are often characterised by intensive signal overlap where coupled nuclei cannot be identified on the basis of simple multiplet peaks [63]. The spectrum obtained from this experiment is set out along the *x*-axis and, in homonuclear COSY, repeated along the *y*-axis and repeated yet again in the contours of the diagonal peaks. Correlation between protons i.e. proton-proton coupling is indicated by the contour of an off-diagonal crosspeak. Spreading the data across an additional axis greatly enhances the ease with which interpretation is possible.

2.3.1.2 NOESY Spectroscopy

NOESY spectroscopy is by far the most important technique in the solution phase conformational analysis of large and complex molecules. The correlation signals i.e. crosspeaks generated from a NOESY experiment result from non-scalar interactions between nuclei which are in a close spatial relationship and are not necessarily coupled to each other. This technique provides, through interatomic

distance information, details of molecular conformation. It thus enables the secondary and tertiary structure of peptides and proteins to be determined [65].

2.3.2 Circular Dichroism Spectroscopy

CD spectroscopy is a sensitive analytical tool used to obtain information regarding the secondary and tertiary structure of peptides and proteins. Basically CD is the differential absorption exhibited by an optically active molecule for left and right circularly polarised light [66]. This technique is particularly sensitive in establishing the conformation of small peptidic molecules, the structure of which may not be as apparent using alternative analytical tools such as NMR in which the complexity of the spectra can sometimes make complete structural elucidation difficult. It was anticipated that the peptides employed in this synthesis would either demonstrate a tendency to adopt α-helical conformations or appear as random coils i.e. unordered.

2.3.3 Molecular Modelling

Computer graphics, or molecular modelling, has become a very powerful tool in the field of drug design and development. This is because it allows theoretical studies of the interaction between a substrate (drug) and receptor (e.g. protein, DNA) of known structure to be modelled visually [67]. The studies obtained from this technique can identify and define the possible key details of the molecular interaction and hence decide on optimal structural modifications likely to enhance either general binding affinity or recognition of specific binding sites. Molecular

modelling, however, is limited in that its results are focused only on the structural and electronic aspects of the actual molecular interaction and does not normally take into consideration factors such as drug absorption, transport and metabolism [67].

The techniques described in this chapter were employed in both the synthesis and evaluation of the peptides. It was anticipated that the biophysical studies would determine the binding activity of the transcription factor inhibitors and that the structural studies would help establish the key features required for maximal biological activity.

3 SYNTHESIS - RESULTS AND DISCUSSION

Synthesis of the potential AP-1 transcription factor inhibitors involved three stages, firstly, synthesis of peptides representing the DNA binding domains of the fos and jun proteins, secondly, attachment of linker/spacer groups to intercalating chromophores (i.e. synthesis of intercalator-linkers) and finally coupling the peptidic moieties to the intercalator-linker groups. This chapter describes and discusses the procedures involved in synthesising the intercalator-linked peptides and the results obtained.

3.1 Peptides

3.1.1 Target Sequence

The tri-amino acid sequence i.e. Lys-Cys-Arg, which is highly conserved in the DNA binding domains of the AP-1 protein was incorporated in the peptides employed in this synthesis. It was anticipated that short, truncated sequences of 5-7 residues bearing this motif would have the potential to bind the DNA AP-1 site in a redox sensitive manner. The Cys residue, essential for its thiol function, was expected to mimic that in the fos and jun proteins, behaving like a redox switch, permitting DNA binding of the drug (in tumour cells) under oxidative stress but preventing binding under oxic conditions. The Cys residue, therefore, was expected not only to determine the DNA binding of these agents, but also to allow them to be selective under reducing/hypoxic conditions.

The Arg and Lys residues, which are basic amino acids, were incorporated to maintain the cationic charge of the peptide, thus making it more electrostatically compatible with the phosphodiester backbone of DNA. As discussed earlier in section 1.3.4, these basic residues also enhance the reactive nature of the Cys residue, making it readily susceptible to oxidation and hence an attractive candidate for redox regulation [29].

3.1.2 Peptide synthesis

Following initial semi-automated synthesis of two peptides, the remaining eight peptides of various amino acid sequences were custom synthesised by Calbiochem-Novabiochem Ltd. Six analogous peptides bearing the KCR motif and two further peptides with RCK and KSR motifs were obtained. These peptides are discussed later in section 3.4. The following section, however, describes the synthesis of the desired peptides using both solid and solution phase techniques.

3.2 Solid Phase Peptide Synthesis

A NovaSyn Gem Peptide synthesiser was employed to synthesise the two peptide sequences shown in figure 19. The method of SPPS adopted was continuous flow, in which the Fmoc strategy [41] was employed, where the α- amino group and the side-chain functionalities of the amino acid residues were protected with base-labile and acid-labile protecting groups respectively.

Sequence I:

FmocAla-**Arg(Pmc)-Cys(Trt)-Lys(Boc)**-Ala.OH

Sequence II:

FmocAla-**Arg(Pmc)-Cys(Trt)-Lys(Boc)**-Ala.NH_2

Figure 19 Peptides Sequences I and II Synthesised using SPPS

Peptides sequences I and II bear the reverse of the desired sequence i.e. RCK as opposed to KCR which is found in the DNA binding domains of the AP-1 protein. These peptides were synthesised to provide suitable controls and to help establish the importance of the KCR motif in the DNA binding of both the AP-1 protein and the peptide. The two peptides have different terminal groups, i.e. an acid and an amide group. The purpose of this was to determine whether the terminal group plays a role in the binding efficiency of the peptide to DNA.

3.2.1 The Peptide Synthesiser

The peptide synthesizer employed was semi-automated, in which the chemical processes for deprotection, washing and acylation were automated. but the preparation and addition of the activated sample and reaction monitoring had to be carried out manually. The flow scheme of a simple computer semi-automated instrument similar to the one employed is shown in figure 20.

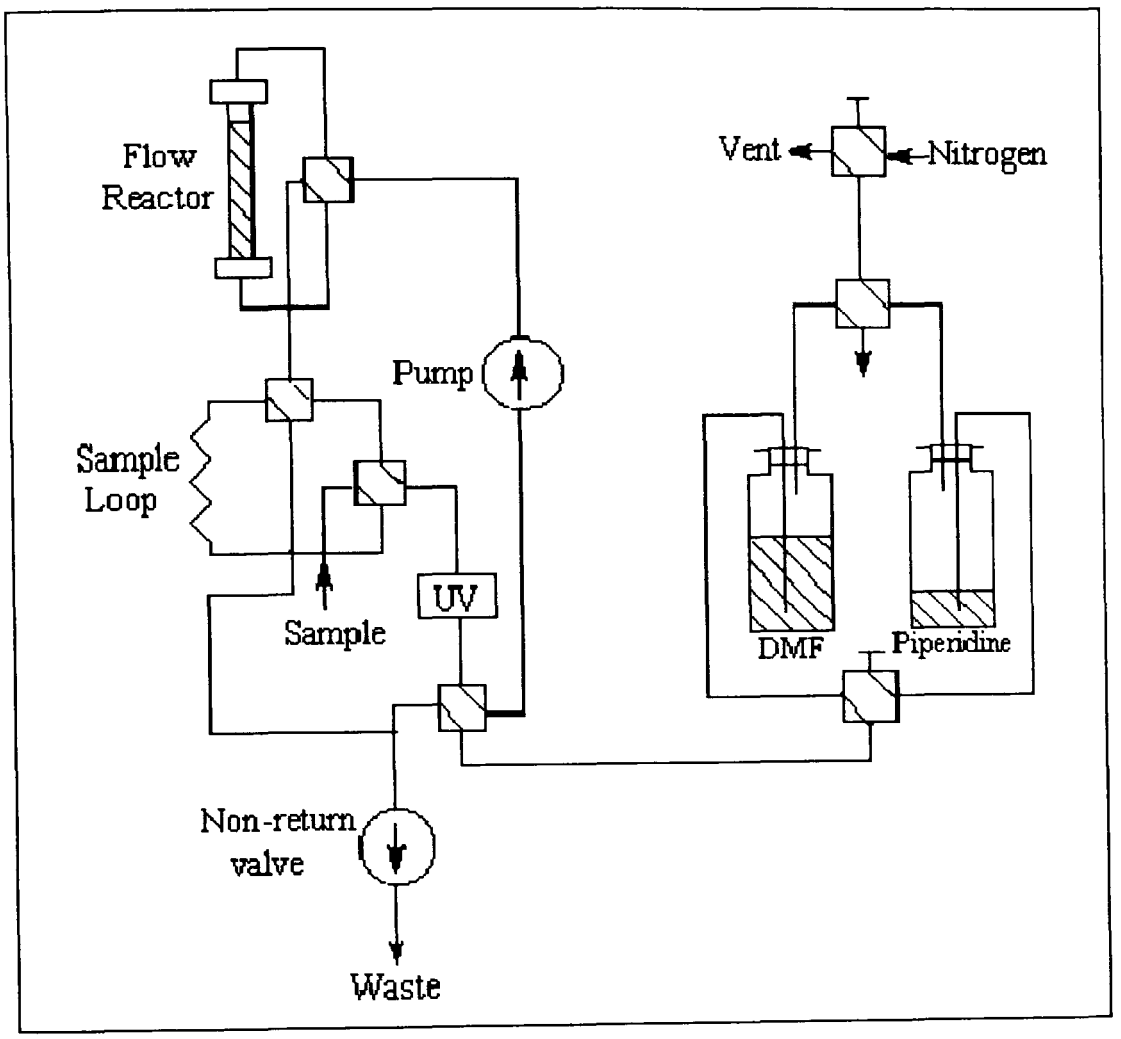


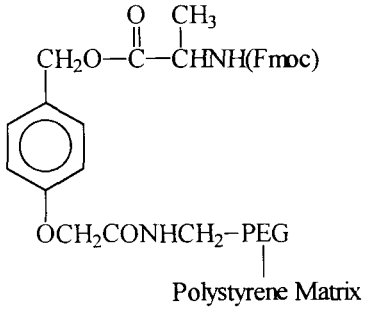
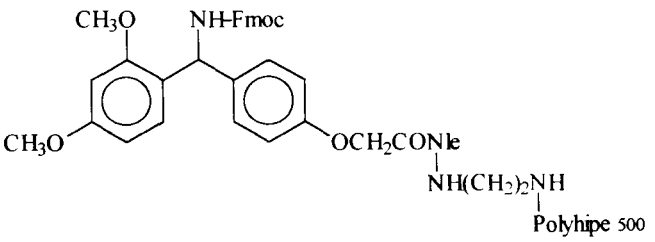
Figure 20 Schematic Diagram of a Semi-automated Peptide Synthesizer

3.2.2 Insoluble Supports and Solvents

The solid supports FmocAla-NovaSyn TGA and NovaSyn PR500 were employed to synthesise the peptide acid and peptide amide respectively. The structures and loading potentials of these resins are shown in table 2. In each case the resins were solvated with dimethylformamide (DMF) for approximately 45min prior to use. This was necessary as the reactions involved in SPPS not only occur on the surface of the polymer but also inside the particles where reactants enter and leave by diffusion. Appropriate swelling of the support was therefore essential for the

maximum access and hence interaction of the reactants with the resin. Product formation was further enhanced by use of DMF, a polar solvent, which increased the solubility of both the N-α protected amino acid residues and the reagents employed. DMF is known to decompose, liberating basic amino functionalities into solution, which are capable of cleaving the Fmoc group of the incoming amino acid residues and hence interfering with the growing peptide. For this reason the DMF used was distilled every 2-3 days.

Table 2 Resins Employed in the SPPS of Sequences I and II (figure 19)

Resin	**FmocAla-NovaSyn TGA**	**NovaSyn PR500 (Polyhipe)**
Structure		
Loading Potential	**0.14mmol/g**	**O.23mmol/g**
Product	**Peptide Acid**	**Peptide Amide**

Nle Norleucine; PEG Polystyrene Graft Polymeric Ethylene oxide

3.2.3 Activation

The coupling agent used in this instance was PyBOP, which is very similar to and therefore often compared with benzotriazole-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP), figure 21, which is an excellent peptide coupling reagent. PyBOP is often used in place of BOP, as it is as efficient and in some cases has shown to cause less racemization. In addition, unlike BOP, it does not form the carcinogenic by-product hexamethyl phosphoramide [68].

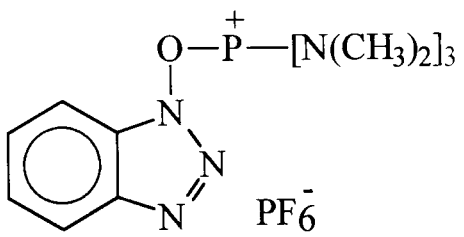
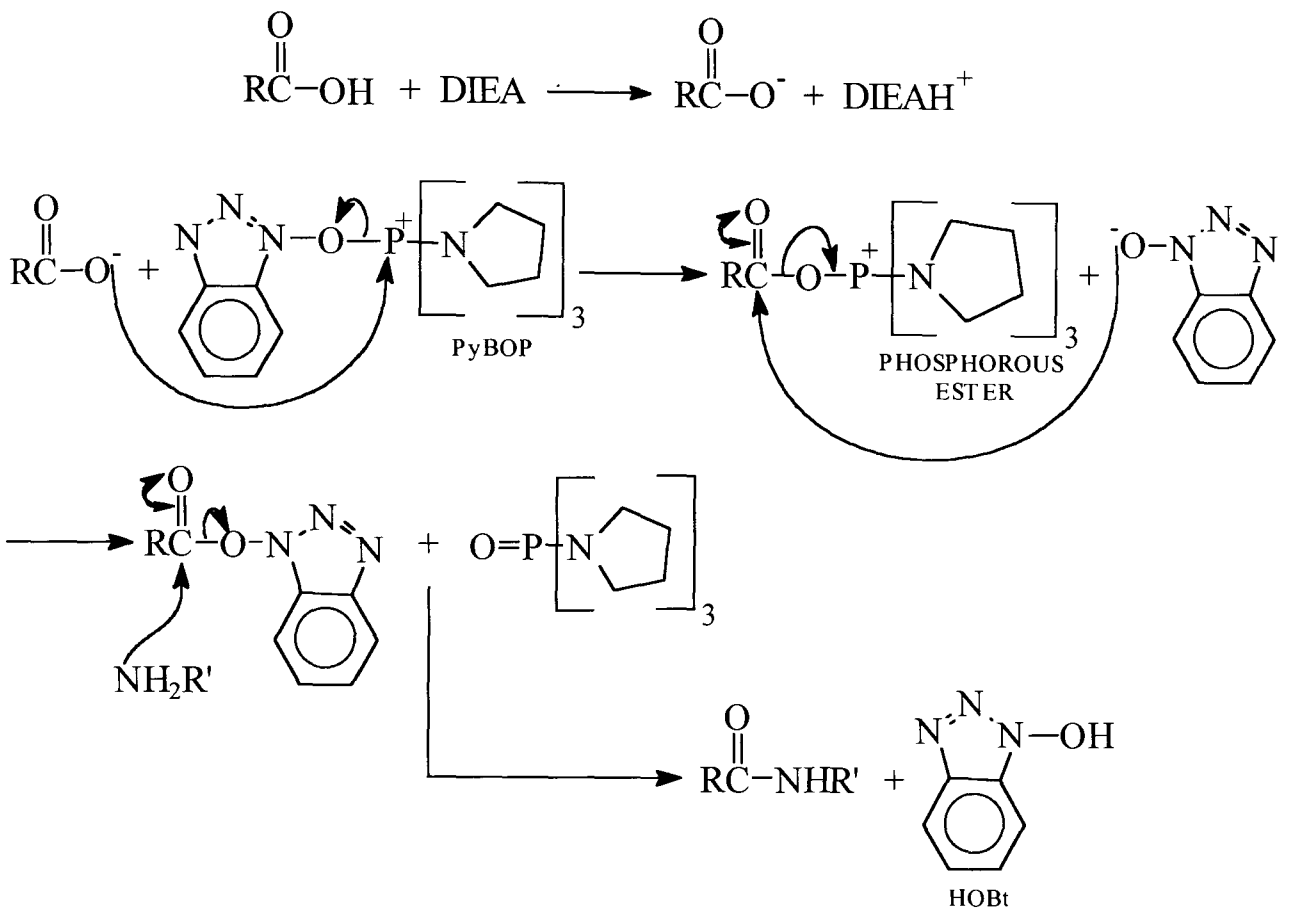


Figure 21 'BOP'

In this reaction (reaction scheme 1) diisopropylethylamine (DIEA) was employed to abstract a proton from the carboxyl group of the amino acid to generate a carboxylate ion. This anion then attacks PyBOP in a nucleophilic displacement reaction, where an amino-phosphorous ester and the auxiliary nucleophile, HOBt, are generated.

Reaction Scheme 1



HOBt behaves like a second nucleophile, destroying the highly activated phosphorous ester and generating the benzotriazolyl ester of the amino acid residue. This is followed by nucleophilic attack of the carbonyl carbon by the amino group of the resin-supported peptide, which results in the formation of the desired product and the generation of HOBt.

3.2.3.1 Acylation Monitoring

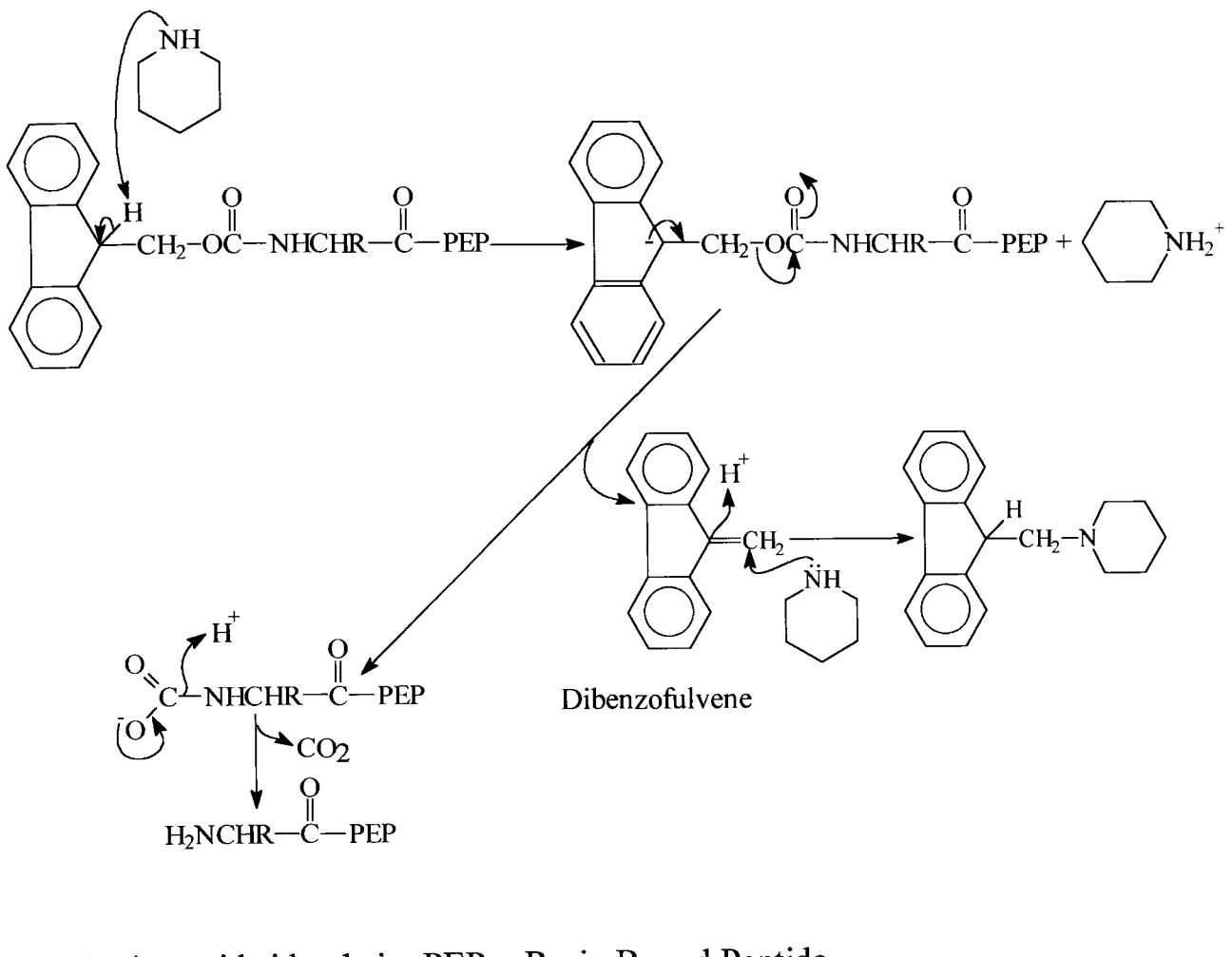
Each acylation step was monitored using the Kaiser test, which is a qualitative colorimetric assay [42]. This essentially involved removing a small aliquot of the resin (3-5mg) from the reaction vessel and treating it sequentially with a few

drops of each of ninhydrin in ethanol, potassium cyanide in ethanol and finally phenol in ethanol at 120°C for a few minutes. The presence of unreacted primary amino groups was indicated by a strong blue-purple colour, indicative of unsuccessful acylation, in which case a further batch of acylating mixture was introduced. A yellow-green colour however indicated that acylation had gone to completion and it was therefore possible to progress to the next stage of the synthesis.

3.2.4 Deprotection

The base labile Fmoc protecting group was removed from the N-terminal amino acid by proton abstraction using 20% piperidine in DMF. This reaction involves the generation of a carbamic acid, which loses carbon dioxide to afford the free amine, as shown in reaction scheme 2.

Reaction Scheme 2



R = Amino acid side-chain; PEP = Resin Bound Peptide

3.2.4.1 Deprotection Monitoring

The progress of each deprotection step was monitored using an UV spectrophotometer. A single absorbance peak identified the cleaved Fmoc group as the dibenzofulvene at approximately 310nm. The UV traces were also indicative of any peptide-chain aggregation, which was characterised by the broadening of the deprotection band. This occurred where there were difficulties of access for the base to the peptide amino terminus and therefore determined the likelihood of encountering difficulties with the subsequent coupling reaction.

When such broadening was observed, a longer acylation period was utilised for coupling the following amino acid.

3.2.5 Final Deprotection and Cleavage

Complete deprotection and cleavage of the peptide from the resin was achieved using TFA (92.5%) and a mixture of scavengers i.e. ethanedithiol (EDT; 2.5%), triisopropylsilane (TIS; 2.5%) and water (2.5%). Scavengers are reagents that trap reactive TFA-liberated carbonium ions and thereby prevent them from undergoing deleterious reactions with sensitive amino acids. The presence of scavengers was particularly important in the TFA deprotection of Cys(Trt) which in the absence of such reagents is reversible. The Trt protecting group is efficiently scavenged by TIS which prevents its reattachment to the Cys residue [69]. EDT, which also assists in the deprotection of Cys(Trt), and water were employed as they are known to accelerate the removal of the Mtr and Pmc protecting groups in TFA [37].

3.2.6 Pentamer Synthesis

The synthesis of the two pentamers followed the protocol shown in figure 22. The NovaSyn TGA resin employed in this synthesis had been pre-loaded with FmocAla-OH, hence the first step shown in the protocol was only applicable to the NovaSyn PR500 resin.

In each case, the amino acid residue was treated with base i.e. DIEA and activated with PyBOP in DMF a few minutes prior to its introduction to the reaction vessel. Acylation, on average took approximately 2hr, however longer coupling times were required for the attachment of the Cys and Arg residues. This was likely to be due to the steric hindrance created by the bulky protecting groups used to protect their side-chain functionalities i.e. the Trt and Pmc groups of Cys and Arg respectively. The time allocated for the removal of the Fmoc group using 20% piperidine in DMF was 20min. The two pentamers were left attached to their respective resins in order to facilitate later reactions involving the attachment of the intercalator-linkers (section 3.6) on the solid phase and also to enable the use of excess reagents.

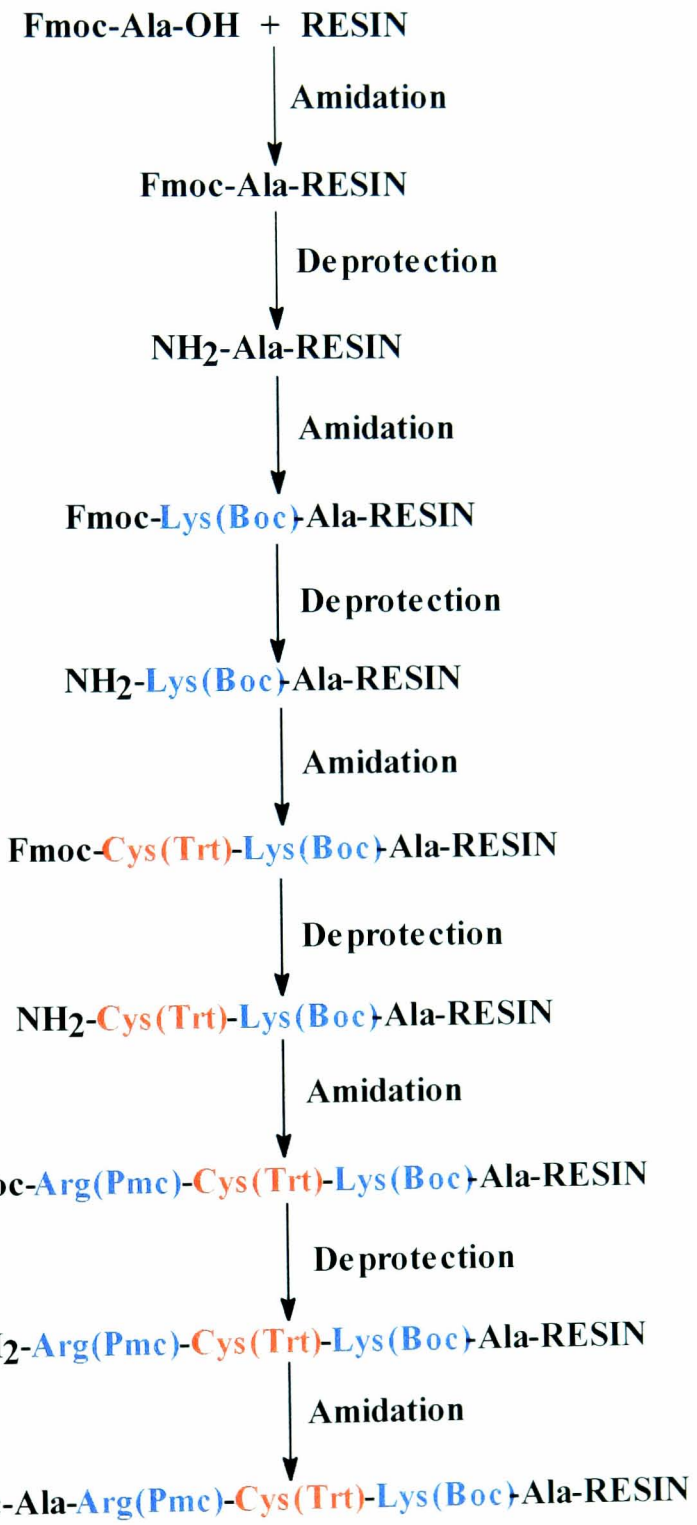


Figure 22 Synthesis of Peptide Sequence I Using SPPS

3.3 Solution Phase Peptide Synthesis

Although SPPS was the favoured method, solution phase synthesis of the peptide (bearing the KCR motif) shown in its protected form, in figure 23, was also attempted.

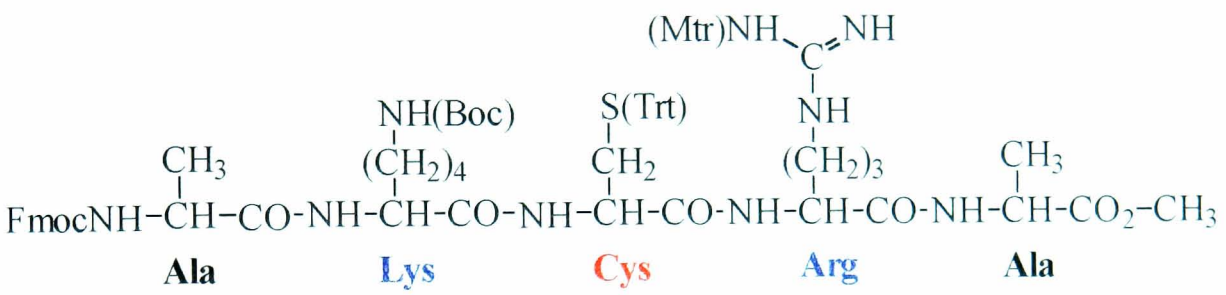
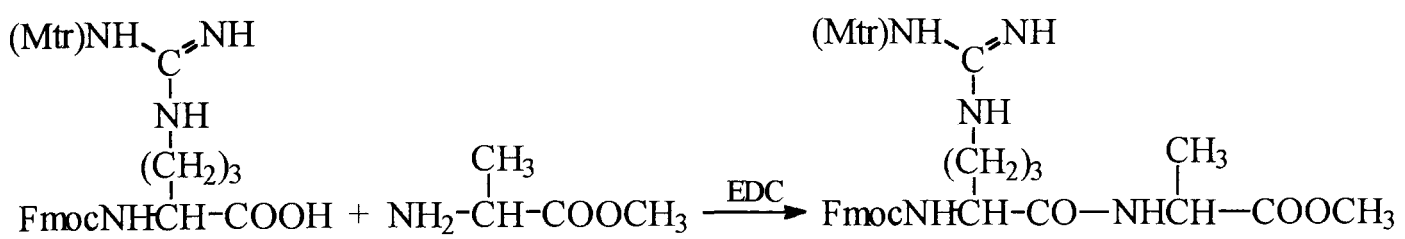


Figure 23 'Protected' Pentapeptide

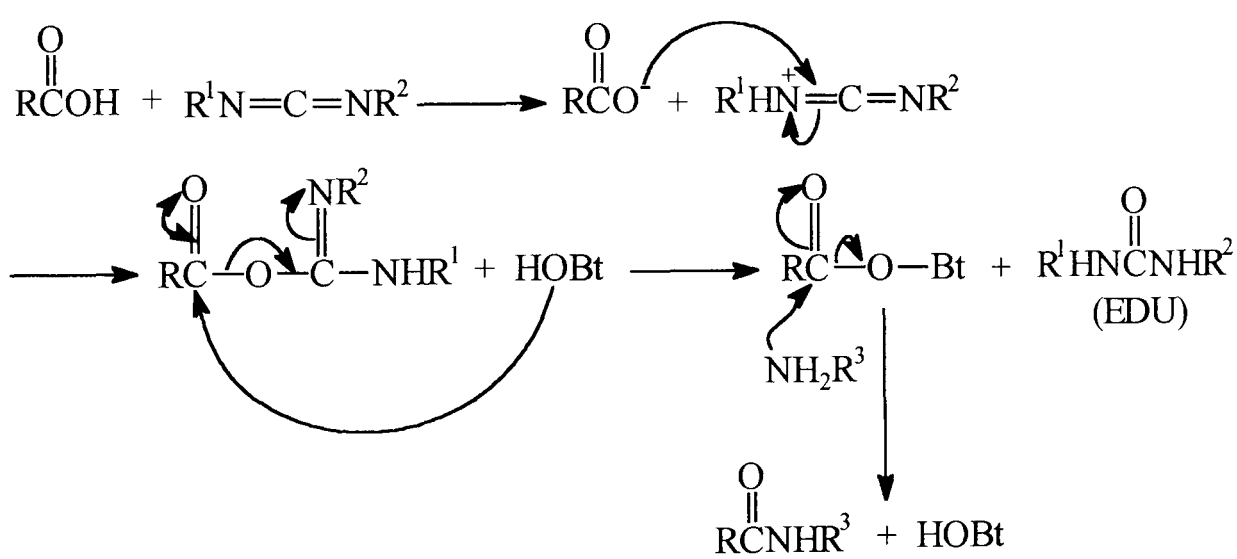
This method of peptide synthesis, however proved to be both difficult and time consuming, this was due to the problems encountered with both the stability and purification of the peptidic intermediates. This instability was found to be more prominent where an ester group had been used to protect the carboxyl function of the amino acid residue. This was especially true of the dipeptide-ester, 'Arg(Mtr)-Ala.OCH_3', which was synthesised using a standard coupling procedure, in which EDC and HOBt were employed, followed by the removal of the Fmoc group using 20% piperidine in dichloromethane.

Reaction Scheme 3

I. Acylation

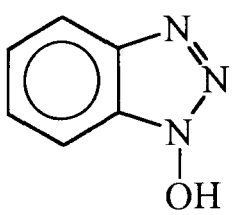


Mechanism

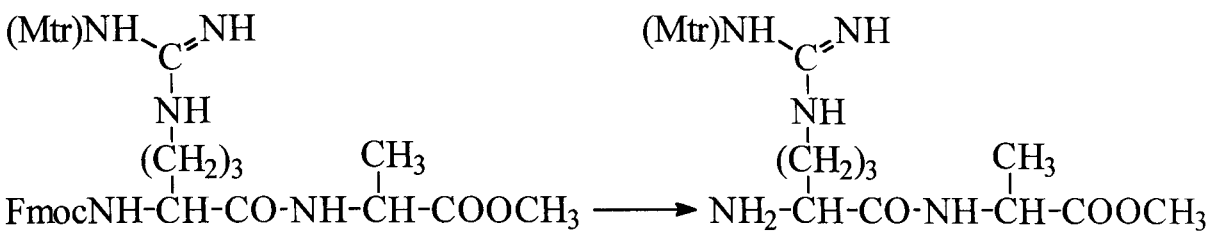


R= FmocNHCH$(CH_2)_3$NHCNHNH(Mtr); $R^1 = CH_2CH_3$; $R^2 = (CH_2)_3NMe_2$;

$R^3 = CH_3CHCO_2CH_3$

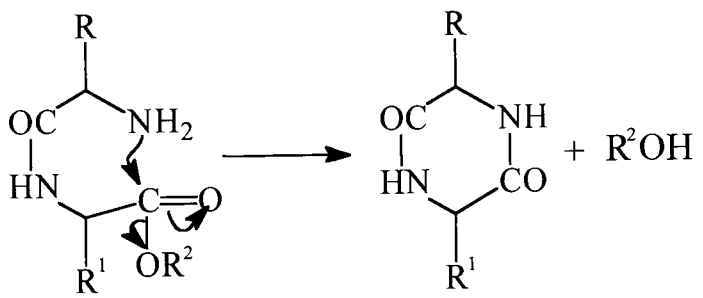
HOBt = 

II. Selective Deprotection



It became apparent from Nuclear Magnetic Resonance (NMR) and Infrared (IR) spectroscopy that the deprotected dipeptide-ester had not been isolated, this was indicated by the absence of the methoxy group of the alanine (Ala) residue. The absence of this group suggested that the deprotected dipeptide-ester was undergoing cyclisation to form a 2,5-diketopiperazine (DKP) ring [37], as shown in reaction scheme 4.

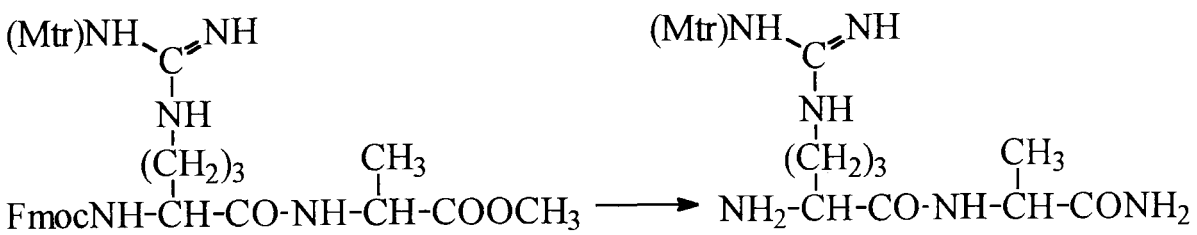
Reaction Scheme 4



R = $(CH_2)_3NHCNHNH(Mtr)$; R^1, $R^2 = CH_3$

In an attempt to avoid DKP formation, the ester function of the Ala residue was transformed to its corresponding amide using (sg 0.88) NH_4OH/CH_3OH. Concomitant deprotection of the Fmoc group was also anticipated.

Reaction Scheme 5



Isolation of this product, however was not successful, and it was likely that transamidation had occurred. Due to both the lack of time and the problems encountered with this method, solution phase peptide synthesis was no longer pursued.

3.4 Peptide Sequences

As mentioned earlier in section 3.1.2, six analogous peptides bearing the KCR motif and a further one with the reverse sequence i.e. RCK, with amide and acid termini respectively were obtained from Calbiochem-Novabiochem. These are shown in figure 24. A control peptide mimicking the binding domains of the oncogenic homologues of the fos and jun proteins, i.e. v-fos and v-jun respectively was also acquired. These oncogene products contain the KSR motif, the serine

residue of which is not subject to redox regulation and thus permits DNA binding of these proteins under both oxidative and reducing conditions [25].

Peptide One

H-Ala-**Arg**-**Cys**-**Lys**-Ala-OH

Peptide Two

H-Ala-**Lys**-**Cys**-**Arg**-Ala-NH_2

Peptide Three

H-Ala-**Lys**-**Ser**-**Arg**-Ala-NH_2

Peptide Four

H-Ala-**Lys**-**Cys**-**Arg**-Asn-Ala-NH_2

Peptide Five

H-Ala-**Lys**-**Cys**-**Arg**-Lys-Ala-NH_2

Peptide Six

H-Ala-**Lys**-**Cys**-**Arg**-Asn-Arg-Ala-NH_2

Peptide Seven

H-Ala-**Lys**-**Cys**-**Arg**-Lys-Arg-Ala-NH_2

Peptide Eight

H-Ala-Ala-**Lys**-**Cys**-**Arg**-Ala-Ala-NH_2

Figure 24 Custom Synthesised Peptides

All eight peptides were synthesised using Fmoc chemistry and were supplied fully protected and bound to resin. The α-amino termini and the side chain functionalities were masked with the base labile Fmoc group and acid labile protecting groups respectively.

3.4.1 Isolation of the Free Peptide

In each case a small amount of the peptide was isolated and analysed in its free form. The purpose of which was to ensure that the desired peptide had been synthesised and that complete deprotection was being achieved. Isolation of the free peptide was achieved by firstly removing the N-terminal Fmoc group using 20% piperidine in DMF. This was then followed by treating the resin with the same cocktail of scavengers discussed earlier in 3.2.5, that is, EDT (2.5%), TIS (2.5%) and water (2.5%) in TFA to simultaneously remove the acid labile protecting groups and to cleave the peptide from its solid support.

3.4.2 Purification of the Peptides

Purification of peptide one was attempted using High Performance Liquid Chromatography (HPLC). A C8 reverse phase column and eluent mixture of water with an increasing gradient of methanol with 1% trifluoroacetic acid was employed. Figure 25 shows the HPLC chromatogram of peptide one prior to purification. Isolation of the pure peptide however was problematical, as the retention time of the desired peak seemed to be variable and analysis of the final product suggested that the purified peptide

was undergoing degradation on standing in the eluent medium. This was indicated by the increase in the number of impurities, i.e. peaks observed in the chromatogram of the final product after purification (figure 26). Due to the problems encountered with the HPLC of peptide one, this method of purification was no longer employed. However in order to eliminate the need for purification and hence minimise the number of impurities the resin-bound peptide was washed thoroughly, at the end of each stage with a range of polar and non-polar solvents.

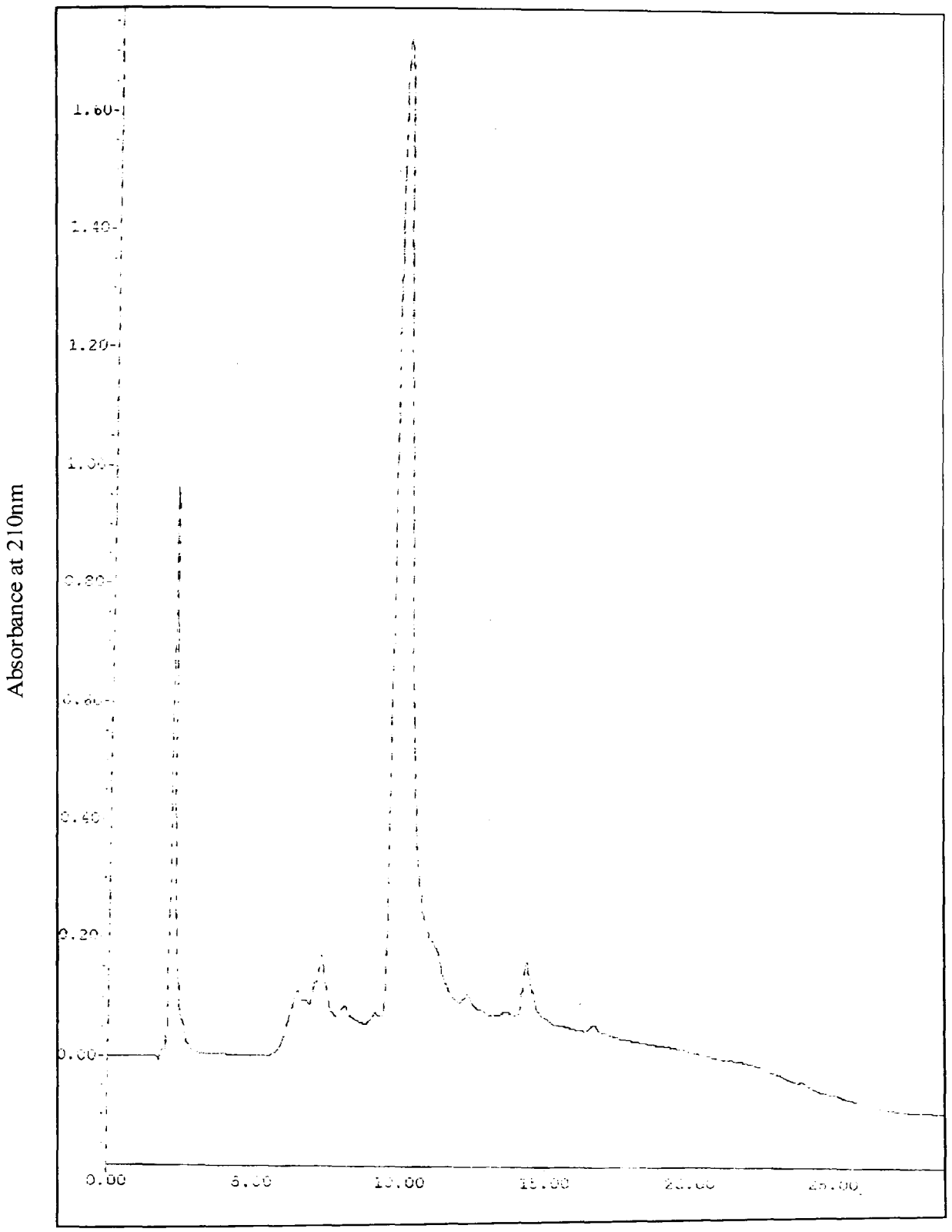


Figure 25 HPLC Chromatogram of Peptide One Prior to Purification Using a C8 Reverse Phase Column and an of Eluent Water with an Increasing Gradient of Methanol

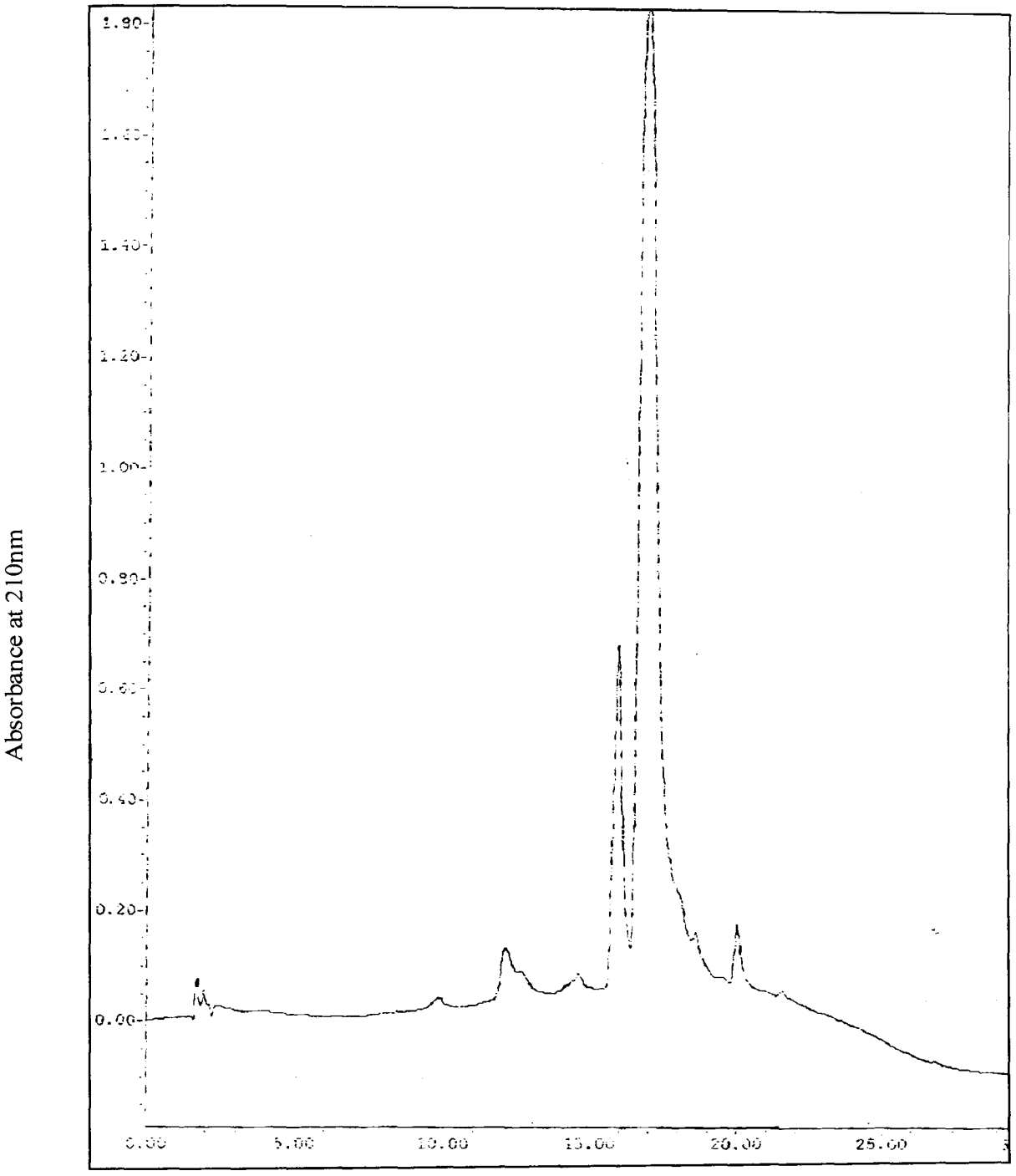


Figure 26 HPLC Chromatogram of Peptide One Post Purification Using a C8 Reverse Phase Column and an of Eluent Water with an Increasing Gradient of Methanol

The crude peptides were analysed using HPLC and Matrix Assisted Laser Desorption Ionisation Mass Spectrometry (MALDI-MS) to ensure that the desired peptides had been isolated. Mass spectral analysis of peptides 1, 5 and 6, however, suggested that the deprotection process was incomplete. This was indicated by the presence of peaks corresponding to the molecular weights of both free and partially protected peptide (figure 27). This suggested that the scavengers employed were not sufficiently suppressing the electrophilic species generated during the cleavage process, which react with the functionalities of sensitive amino acid residues. In order to ensure complete deprotection of the peptides, the amount of each scavenger and the time allowed for the process was increased from 2.5% and 4 hours to 5% and 5 hours respectively. The results hereafter indicated that complete deprotection of the peptides was being achieved (see figure 28).

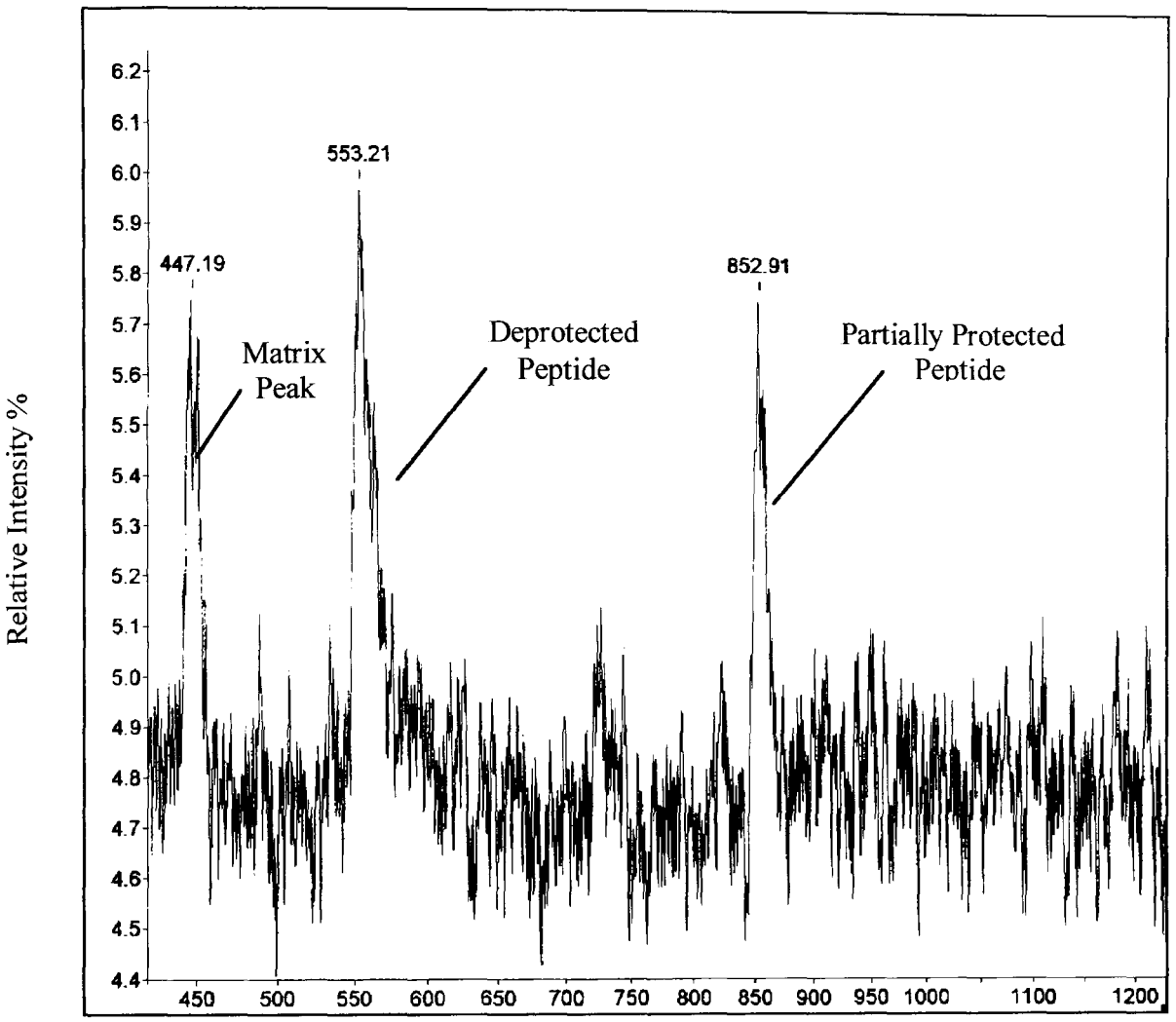


Figure 27 MALDI Mass Spectrum of Peptide One after Deprotection and Cleavage from Resin using EDT (2.5%), H_2O (2.5%) and TIS (2.5%) in TFA

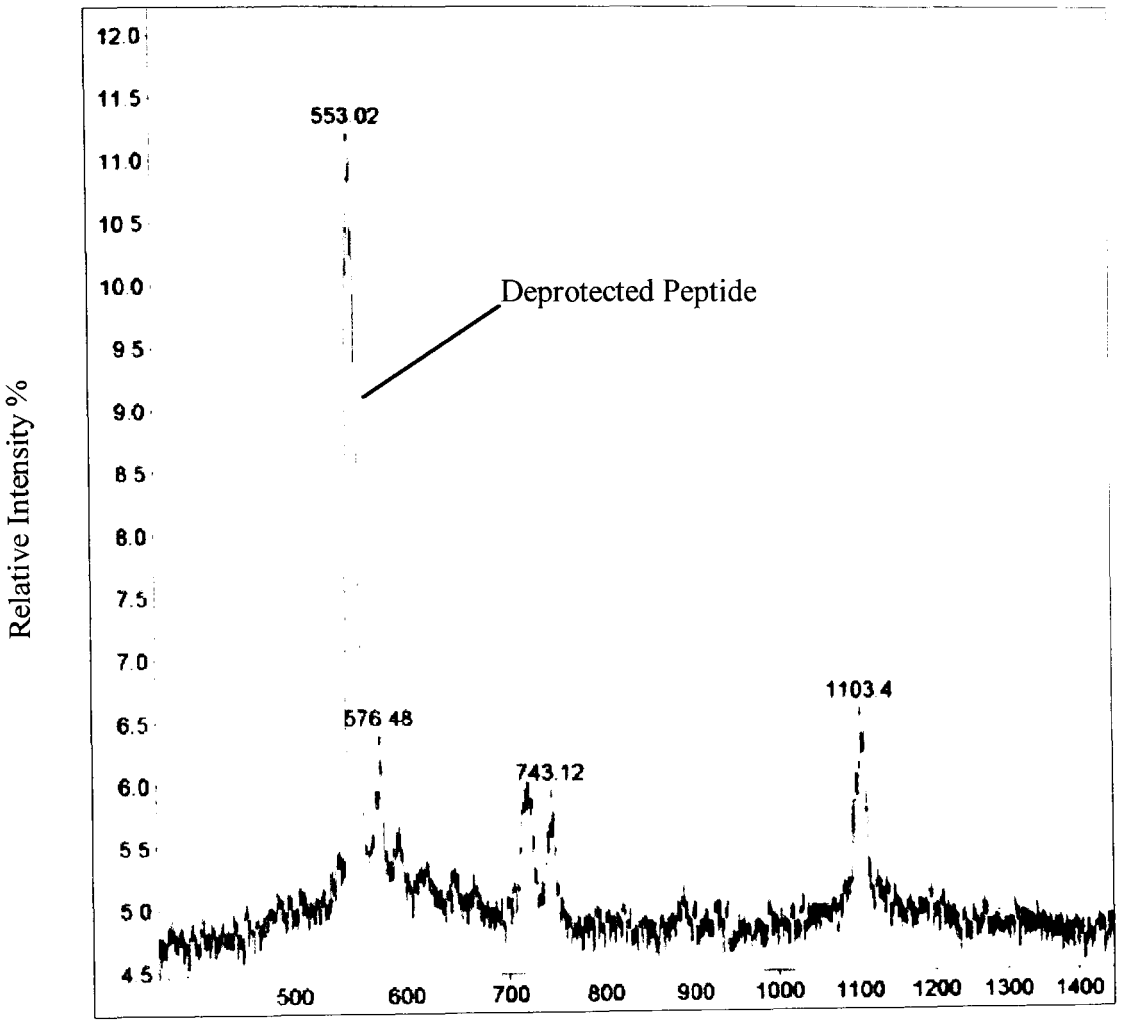


Figure 28 MALDI Mass Spectrum of Peptide One after Deprotection and Cleavage from Resin using EDT (5.0%), H_2O (5.0%) and TIS (5.0%) in TFA

3.5 Intercalator-Linkers

3.5.1 Intercalator

The role of the intercalator is essentially to deliver the peptide hybrid to nuclear DNA. It was envisaged that the non-specific binding of the intercalating chromophore would also stabilise the weaker specific binding of the peptide, the whole providing both high DNA affinity coupled with sequence selectivity. Unfortunately, however, as the chromophore stacks between the adjacent base pairs of DNA it causes local unwinding of the DNA [32]. This distortion of the double helix at the site of intercalation may therefore disrupt DNA binding of the peptide moiety of the conjugate. For this reason a 'spacer' or 'linker' group was employed to distance the peptide and intercalator binding sites.

3.5.2 Linker

In addition to providing a spacer to allow minimal disruption of the AP-1 binding domain by intercalation of the chromophore, the linker group was incorporated in order to ascertain a spatial arrangement which allowed the unrestricted or unhindered movement of the peptide with respect to the intercalated chromophore. It was anticipated that this would effectively allow the peptide to search for the DNA AP-1 site. A number of linker groups of various lengths were investigated in order to determine the

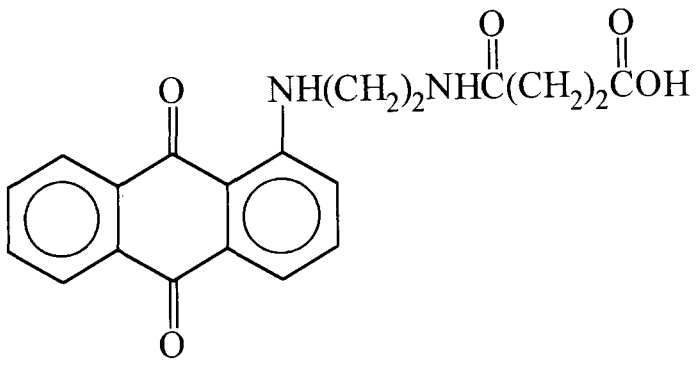
ideal/optimal length necessary to provide the maximum spatial orientation required to ensure the binding of the peptide with the DNA AP-1 site.

3.5.3 Intercalator-Linker Synthesis

The next section describes the procedures involved in attaching various linker moieties to desired intercalators. The intercalator primarily employed in this synthesis was anthraquinone, although the acridine and anthrapyrazole chromophores were also explored. The anthrapyrazoles however were investigated by co-workers [70,71]. The termini of the linkers employed were either carboxyl or chloro groups, in order that attachment of the desired peptides might be achieved using standard acylation and alkylation conditions.

3.5.3.1 Synthesis of 1-[*N*-{2-Succinamidylethyl}amino]anthraquinone (A)

Product

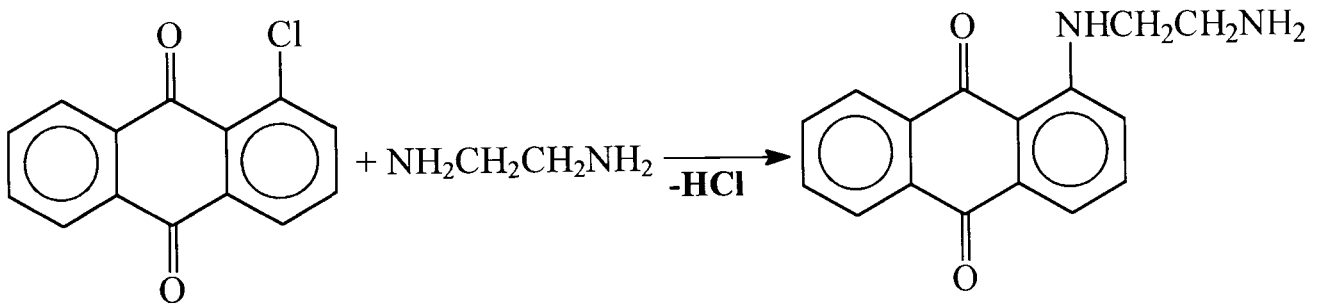


Preparation of 1-[*N*-{2-succinamidylethyl}amino]anthraquinone entailed a two step process. The first reaction involved a nucleophilic displacement reaction in

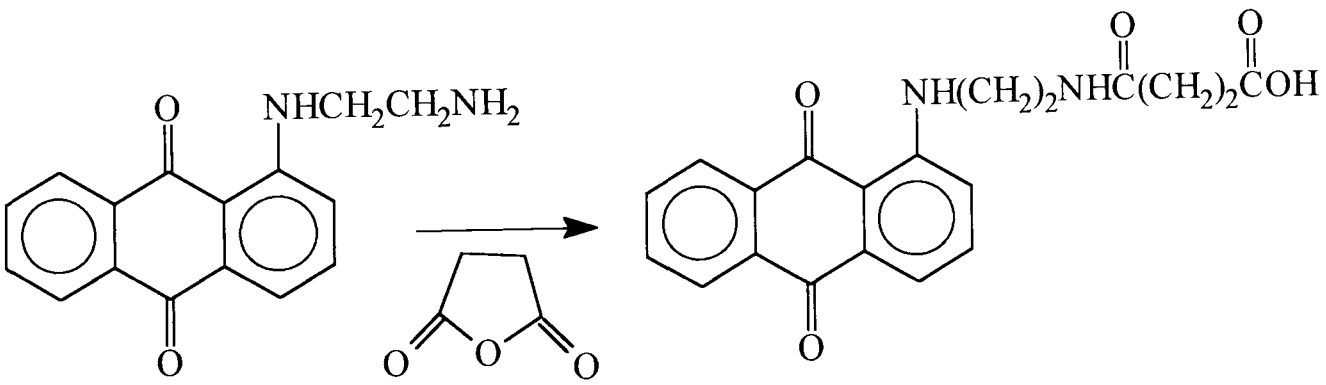
which 1-chloroanthraquinone was reacted with excess ethylenediamine to yield 1-[*N*-{2-aminoethyl}amino]anthraquinone. This was then acylated with succinic anhydride in DMF to afford the desired product in a 72% yield. The products in both cases were purified using column chromatography.

Reaction Scheme 6

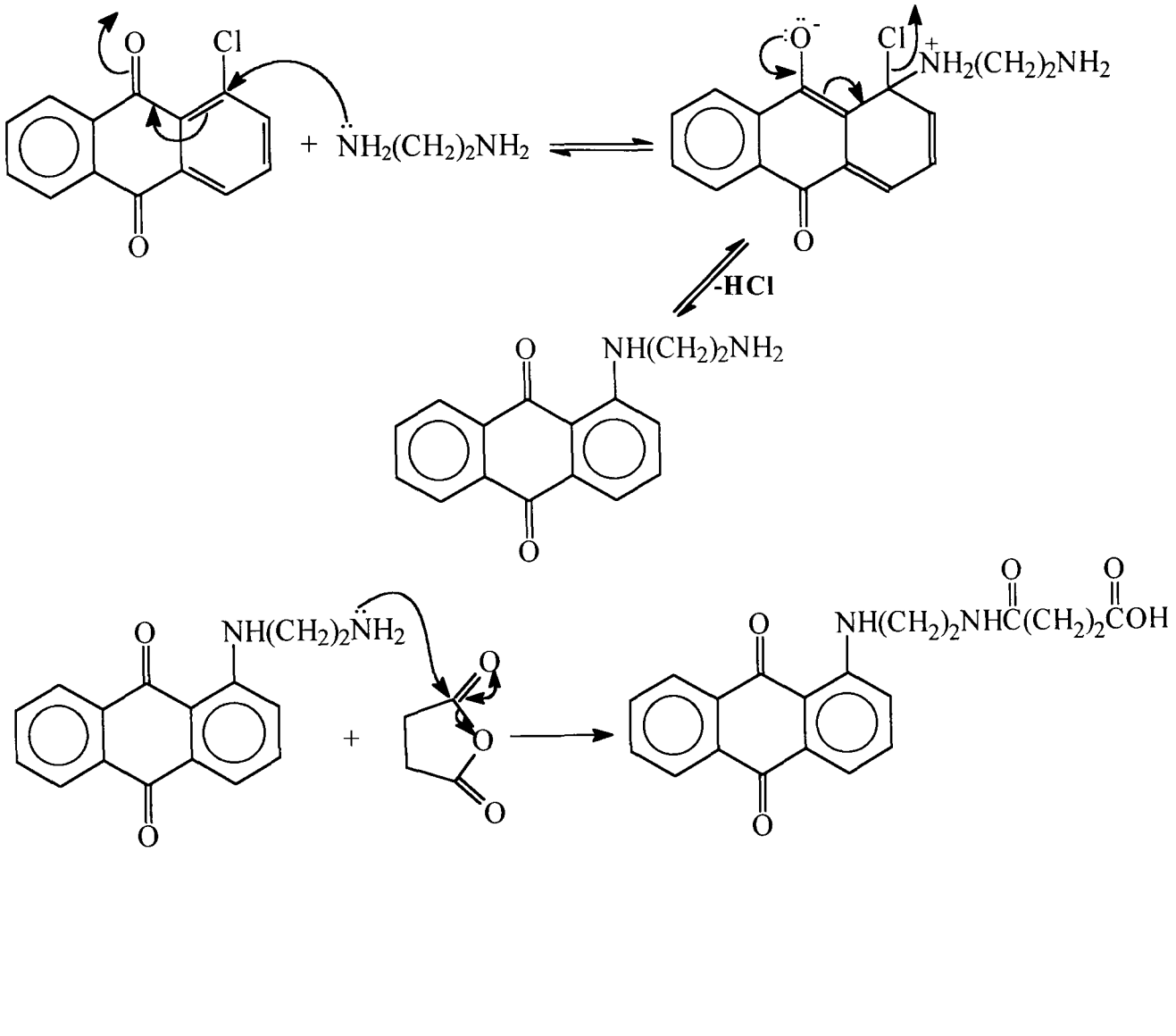
I Amination



II Amidation

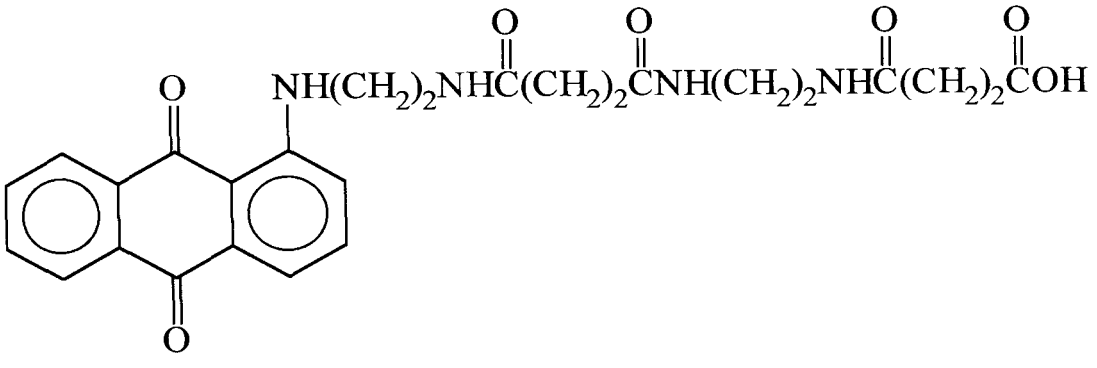


Mechanism


O
Cl
+ NH2(CH2)2NH2
O
:O:-
Cl
+
NH2(CH2)2NH2
O
-HCl
O
NH(CH2)2NH2
O
O
NH(CH2)2NH2
+
O
O
O
O
NH(CH2)2NHC(CH2)2COH
O
O

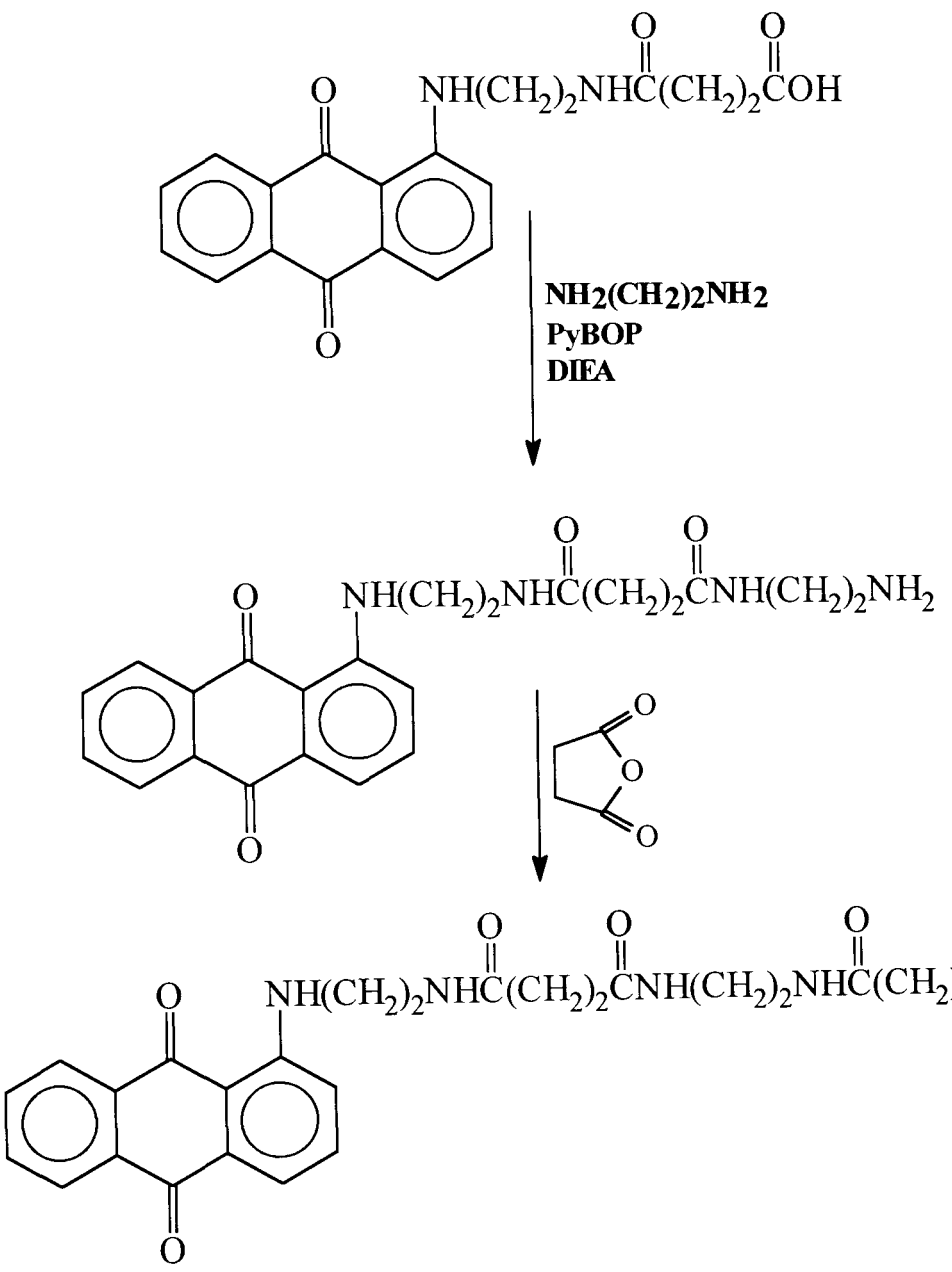
3.5.3.2 Synthesis of 1-[*N*-{2-(2-Succinamylethyl)succinamidyl}ethyl]amino-anthraquinone (B)

Product



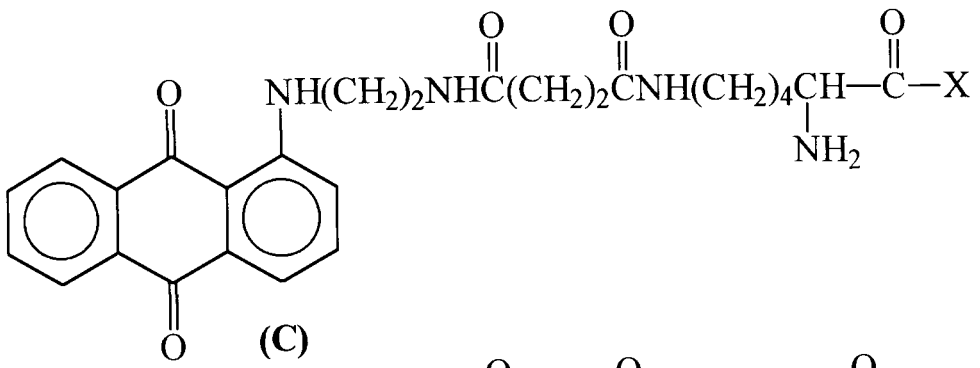
Intercalator-linker B is essentially an extension of A and very similar to C (section 3.5.3.3) except that unlike the latter it does not posses any free amino groups which are required for the formation of non-specific salt bridges. The linker length in B was increased from 8 atoms in A to 16 by repeating the addition of the linker twice. The carboxyl function of A was activated with PyBOP, in the presence of DIEA, and then amidated with excess ethylenediamine, in an attempt to prevent dimerisation, to afford 1-[2-{*N*-(2-succinamylethyl)succinamidyl}-ethylamino]anthraquinone. This was further reacted with succinic anhydride to yield intercalator-linker B in a 25% yield. In both cases the products were purified using column chromatography.

Reaction Scheme 7

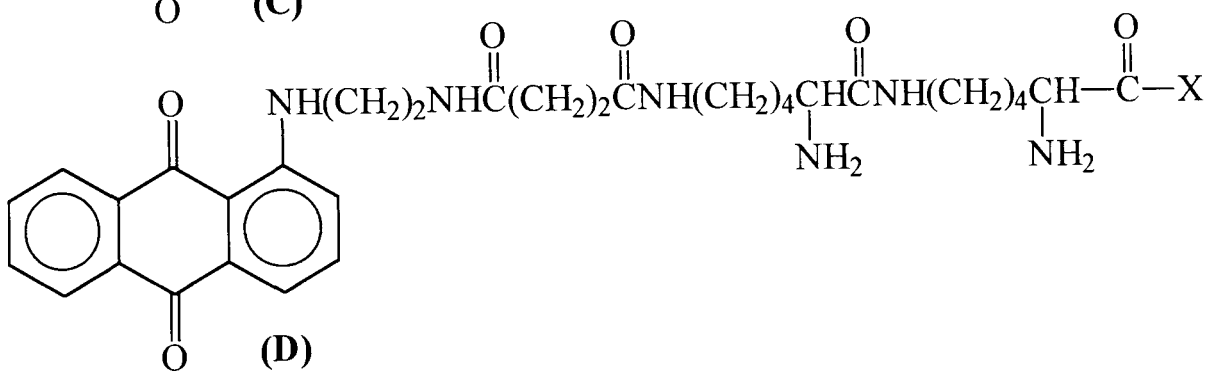


3.5.3.3 Synthesis of 1-N-[2-{*N*-[(5,5)-5-Amino-5-carboxypentyl]succinamyl}-ethyl]aminoanthraquinone (C) and 1-N-[2-{N^1-[(5,5)-5-Amino-5-N^2-{(5^1,5^1)-5^1-amino-5^1-carboxypentyl}carboxamidylpentyl]succinamyl}amino-anthraquinone (D)

Products



(C)



(D)

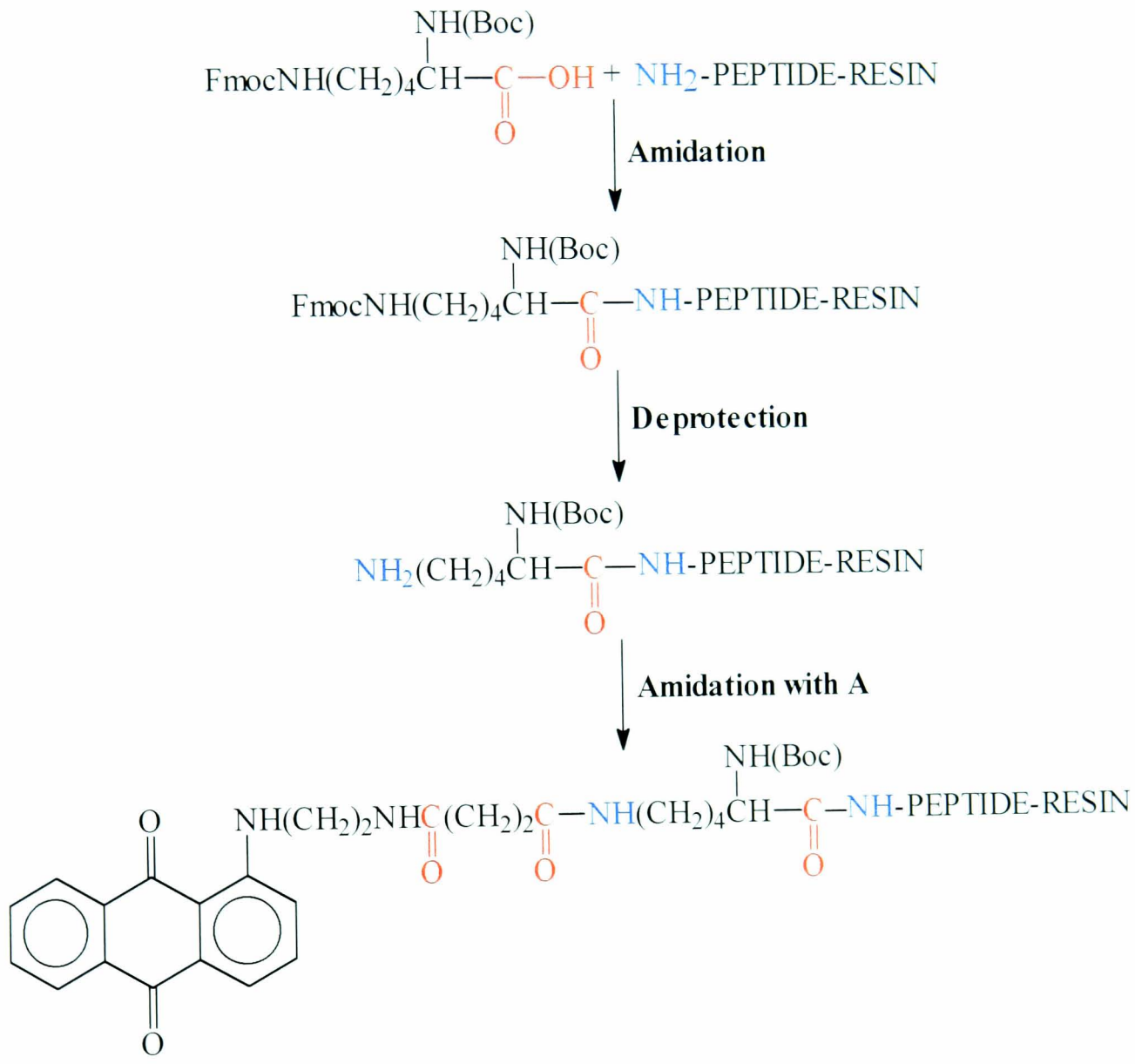
X = Peptide

Intercalators C and D are also extended forms of intercalator-linker A. The linker length was increased from 8 to 15 atoms using a single Lys and from 8 to 22 atoms using two Lys residues (connected through the ε-amino groups) for compounds C and D respectively. This was achieved by linking the Lys residue(s) directly onto the peptide after which the intercalator moiety i.e. A was attached. It was anticipated that the pendant primary amino group(s) present in

the linker would increase the range of non-specific contacts with DNA and hence would further enhance the affinity of these conjugates for DNA.

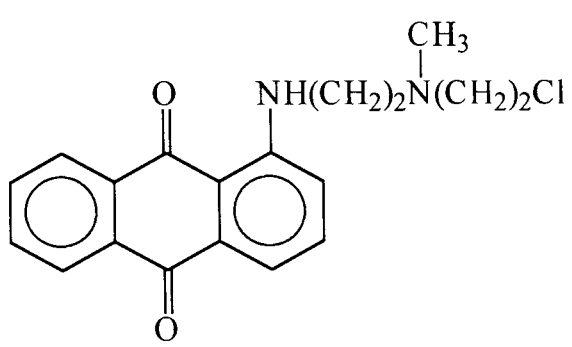
In order to achieve this extension of the peptides by lysyl residues, orthogonal amino protecting groups were required. The ε-amino group of BocLys-OH was protected using 9-fluorenylmethyloxycarbonyl-N-hydroxysuccinimide (Fmoc-OSu) to afford BocLys(Fmoc)OH. This was then used to acylate the terminal amino group of a resin bound peptide using PyBOP. The Fmoc group was then selectively removed and the ε-amino group was subsequently amidated with A to afford conjugate C. In the case of D, a second lysine residue was incorporated before finally attaching A. The synthetic route employed to synthesise conjugate C is shown in reaction scheme 8.

Reaction Scheme 8



3.5.3.4 Synthesis of 1-[2-{*N*-(2-Chloroethyl)-N-methylamino}ethylamino]-anthraquinone (E)

Product



Synthesis of the 1-[2-{*N*-(2-chloroethyl)-N-methylamino}ethylamino]-anthraquinone (E) involved three main steps [72]. The first of which entailed the preparation of the amino-alcohol linker group, i.e. 2-{N-(2-hydroxyethyl)-N-methylamino}ethylamine. The synthesis of this side-chain was attempted using two different methods which have been described below.

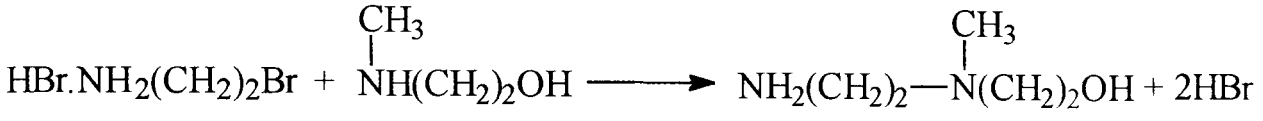
Reaction I Synthesis of 2-[*N*-{2-Hydroxyethyl}-N-methylamino]ethylamine

Method A

Method A was initially chosen for its simplicity as it involved a one step procedure in which neither of the amino components used required protection (see reaction scheme 9). This method essentially involved refluxing bromoethylamine hydrobromide in excess 2-(*N*-methylamino)ethanol for 5hr. Although purification of the product isolated was attempted using kugurohr distillation, Thin Layer Chromatography (TLC) of the resultant yellow oil indicated that the desired

amino ethanol side-chain was present only in a small proportion amongst a number of impurities including 2-(*N*-methylamino)ethanol. Due to the difficulties in isolating pure 2-[*N*-{2-hydroxyethyl}-N-methylamino]ethylamine an alternative strategy was adopted in which amino protection was employed.

Reaction Scheme 9

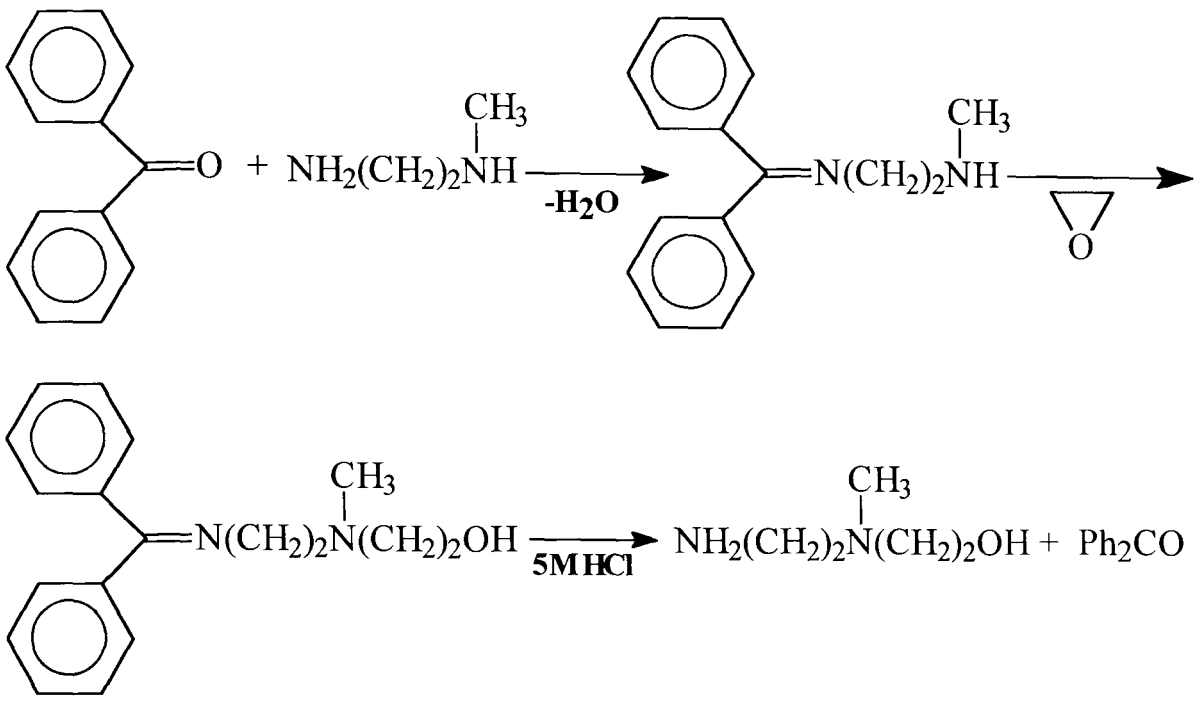


Method B

Synthesis of the amino-ethanol side chain using this procedure involved three stages [72]. In the first step the primary amino group of N-methylethylene-diamine was selectively protected with benzophenone, the carbonyl carbon of which undergoes nucleophilic attack with primary amines, followed by dehydration to form the corresponding imine. The reactants were refluxed in xylene, a high boiling solvent, using a Dean-Stark apparatus to remove the water generated during the course of the reaction. The reaction was performed in the presence of boron trifluoride etherate, a Lewis acid, to enhance the electrophilic nature of benzophenone, making it more readily susceptible to nucleophilic attack. The product was subsequently treated with ethylene oxide, which underwent base catalysed ring opening to give the desired product. Removal of the benzophenone protecting group was achieved by protonation of the imine, making it susceptible

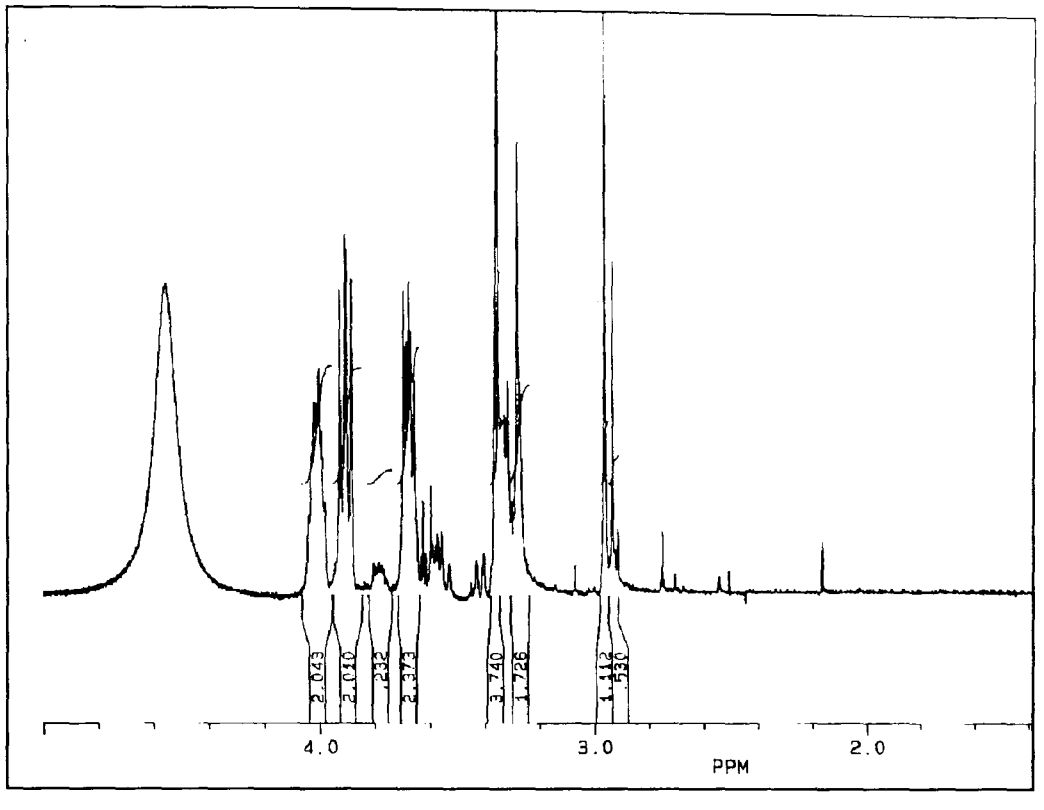
to hydrolysis, using 5M hydrochloric acid. The desired product was isolated using kugurohr distillation.

Reaction Scheme 10



Comparisons of the two products using TLC and NMR (figure 29) indicated that the amino-ethanol side-chain synthesised using method B was more pure than that isolated from method A. The greater purity of the product isolated from method B was due to the extremely volatile nature of ethylene oxide, the removal of which was easily achieved compared to that of 2-(*N*-methylamino)ethanol used in method A. This procedure was therefore adopted hereafter.

(i)



(ii)

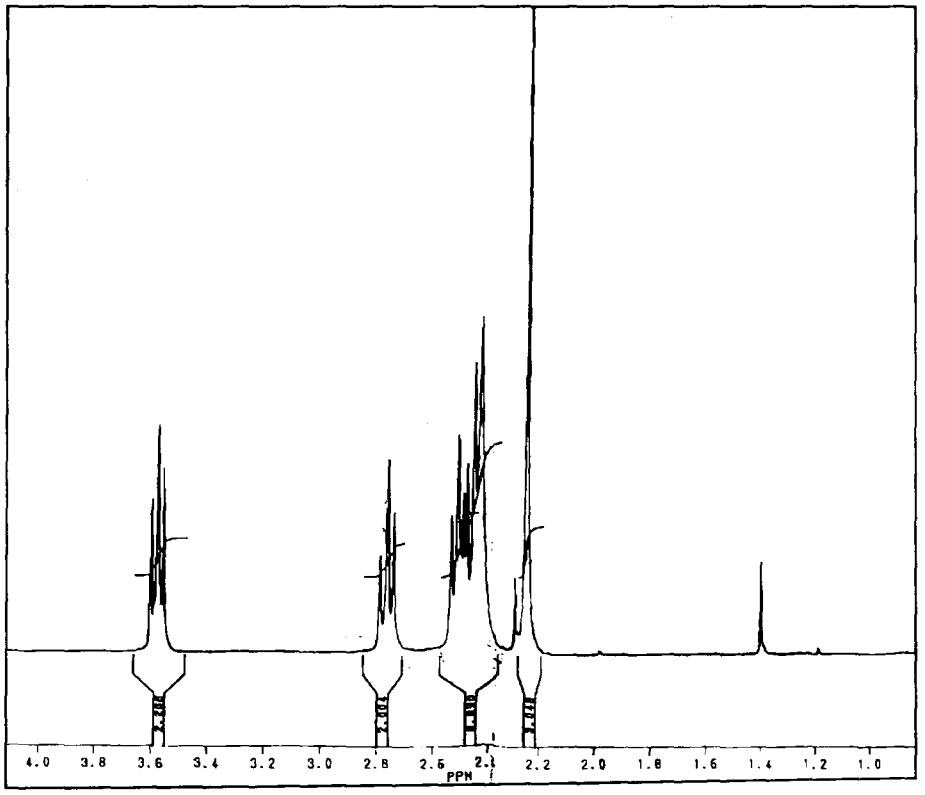
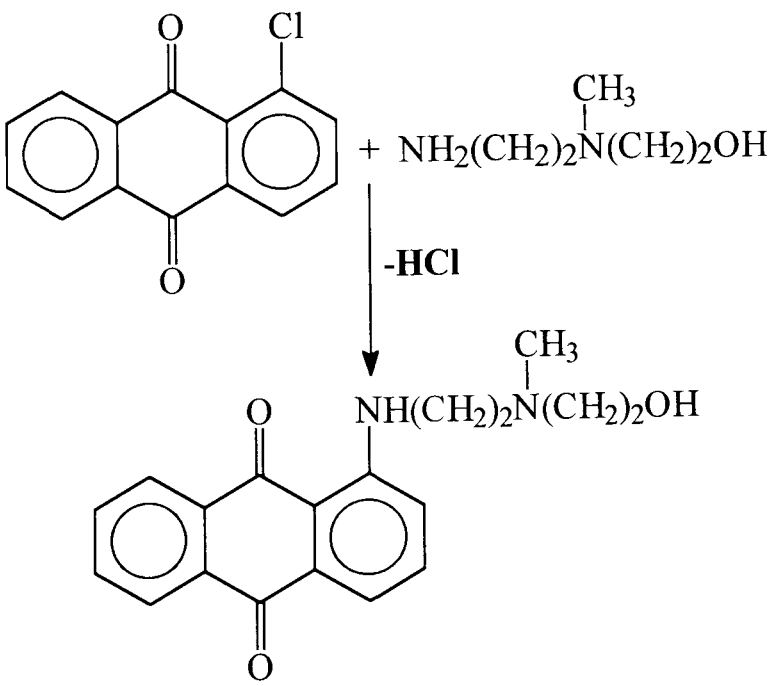


Figure 29 NMR Spectra of* 2-{N-(2-Hydroxyethyl)-N-methylamino}ethyl-*amine Isolated from Method A (i) and Method B (ii)

Reaction II Synthesis of 1-[2-{*N*-(2-Hydroxyethyl)-N-methylamino}ethyl-amino]anthraquinone

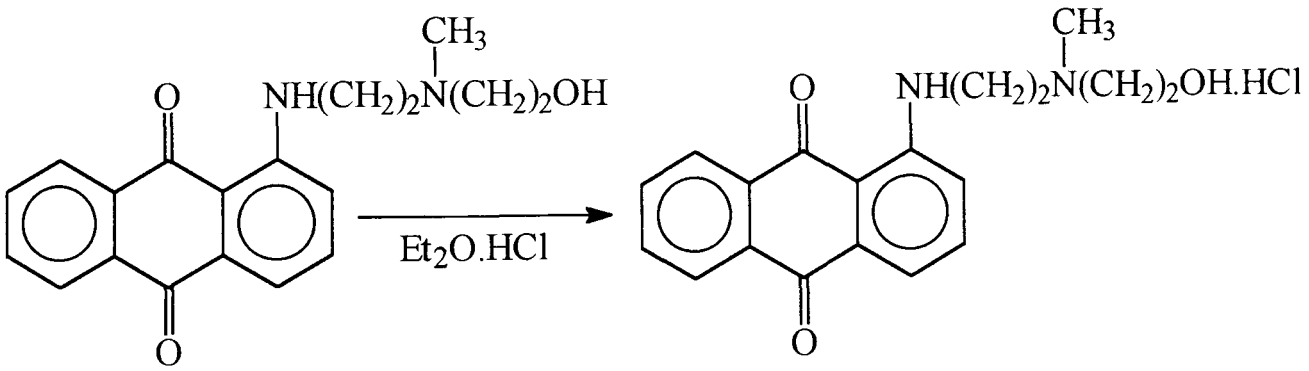
Attachment of the amino ethanol side-chain to the intercalating chromophore was achieved by refluxing 1-chloroanthraquinone in excess 2-[*N*-{hydroxyethyl}-N-methylamino]ethylamine under nitrogen for 18hrs. The product was isolated as a red solid with two major impurities, which were unreacted 1-chloroanthraquinone and the amino-ethanol side-chain. A 12% yield of the desired product was isolated after purification, using column chromatography. The low yield obtained for this reaction was attributed to the poor electrophilic nature of 1-chloroanthraquinone, which was used in favour of the more reactive 1-fluoroanthraquinone for its commercial availability.

Reaction Scheme 11



The hydrochloride salt of 1-[2-{*N*-(2-hydroxyethyl)-N-methylamino}ethylamino]-anthraquinone was prepared in order to prevent the amino groups from interfering in the subsequent chlorination reaction. This was necessary as it had previously been shown by HPLC [72] that chlorination of 1-[2-{*N*-(2-hydroxyethyl)-N-methylamino}ethyl-amine]anthraquinone without prior hydrochlorination resulted in multiple products. Preparation of the salt was achieved using ethereal hydrogen chloride. Addition of the latter solution to 1-[2-{*N*-(2-hydroxyethyl)-N-methylamino}ethylamine]anthraquinone in dichloromethane resulted in the formation of a precipitate and a colour change from red to orange indicating protonation of the secondary amine.

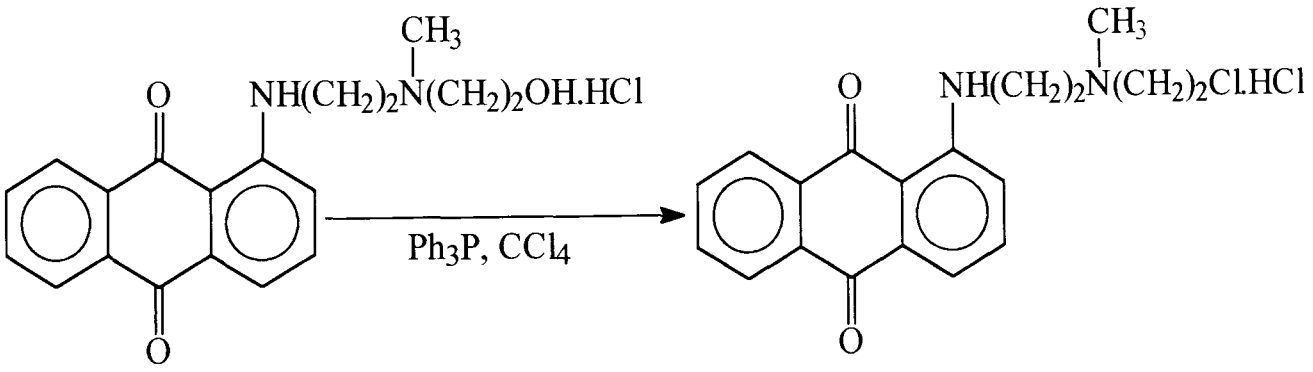
Reaction Scheme 12



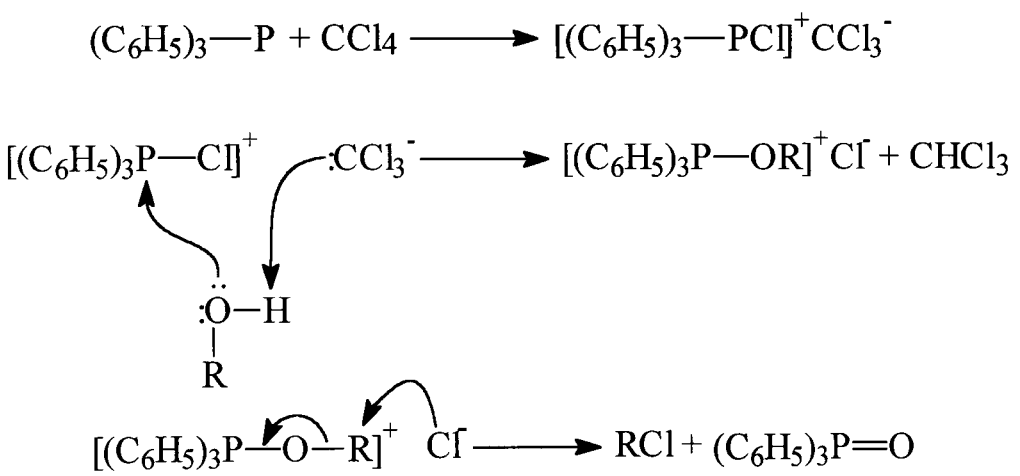
Reaction IV Synthesis of 1-[2-{*N*-(2-Chloroethyl)-N-methylamino}ethyl-amino]anthraquinone

Thionyl chloride was initially used to transform 1-[2-{*N*-(2-hydroxyethyl)-N-methylamino}ethylamine]anthraquinone to its corresponding chloroethyl (mustard) derivative. However the product was isolated in low yield (9%) and hence an alternative method was employed in which the hydroxy derivative was treated with triphenylphosphine and carbon tetrachloride in dichloromethane [73]. The product was purified by column chromatography to remove the residual triphenylphosphine and triphenylphosphine oxide, and was isolated in a 68% yield. The greater yield obtained with this method was attributed to the milder chlorination conditions provided by triphenylphosphine and carbon tetrachloride reagents compared to thionyl chloride, the use of which resulted in the formation of a number of by-products.

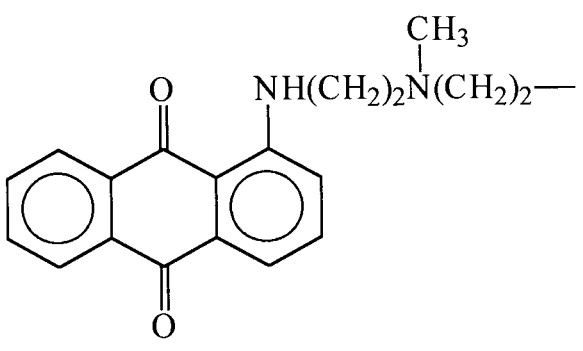
Reaction Scheme 13



Mechanism

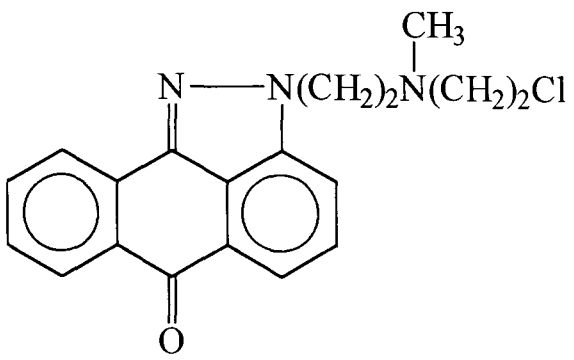

(C6H5)3—P + CCl4
[(C6H5)3—PCl]+CCl3-
[(C6H5)3P—Cl]+
:CCl3-
[(C6H5)3P—OR]+Cl- + CHCl3
:O—H
R
[(C6H5)3P—O—R]+
Cl-
RCl + (C6H5)3P=O

R =


CH3
O
NH(CH2)2N(CH2)2—
O

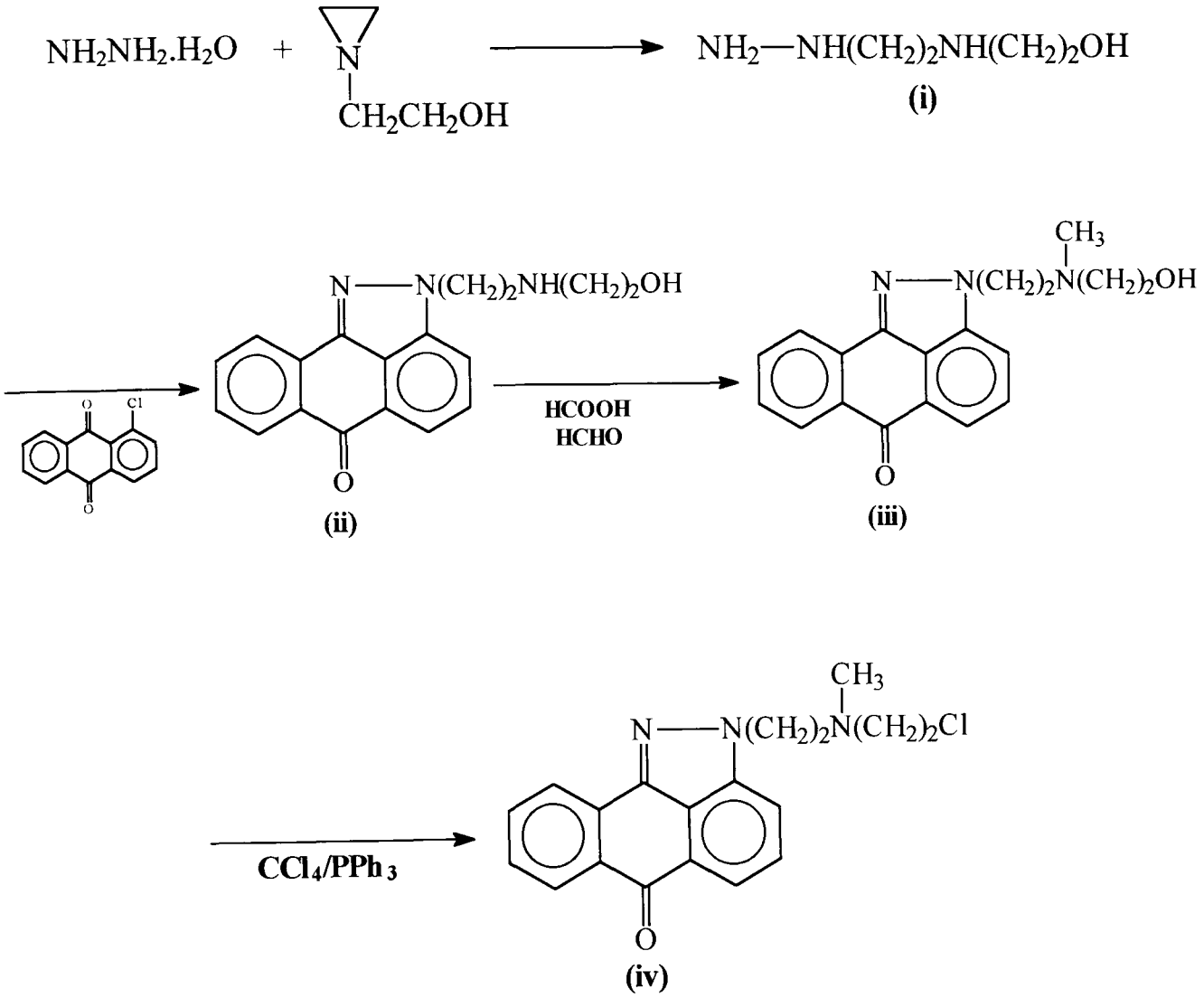
3.5.3.5 Synthesis of 2-[2-{*N*-(2Chloroethyl)-N-methylamino}ethylamino]-anthra[1,9-*c,d,*]pyrazol-6(*2H*)one (F) [70,71]

Product



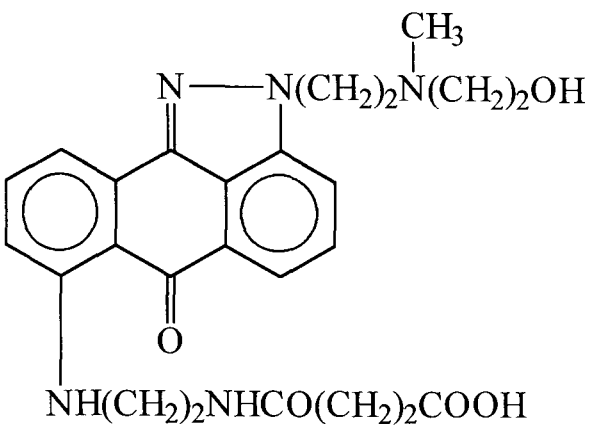
An anthrapyrazole intercalator was employed as an alternative to anthraquinone to determine the effect of the chromophore structure on the DNA binding of the peptide. Synthesis of this intercalator-linker involved four reactions, the first of which entailed refluxing aziridineethanol in hydrazine monohydrate, the former of which underwent nucleophilic attack to afford substituted hydrazine (i). This was treated with 1-chloroanthraquinone, in the presence of triethylamine, in acetonitrile to yield an anthrapyrazole (ii), which was subsequently methylated using formic acid and formaldehyde. The product (iii) was chlorinated using carbon tetrachloride and tiphenylphosphine, as described in section 3.5.3.4, to afford the desired product (iv) in a 57% yield after recrystallisation from hot methanol.

Reaction Scheme 14



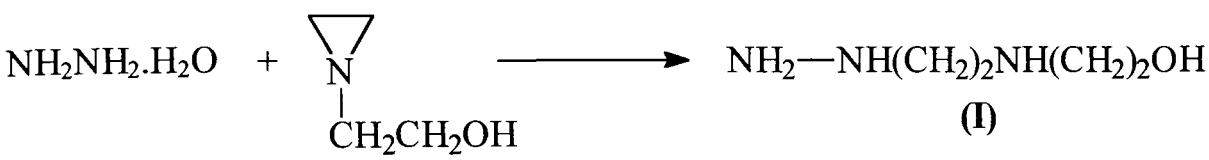
3.5.3.6 Synthesis of 7-[2-{Succinamidoethyl}amino]-2-[2-{*N*-(hydroxyethyl)-N-methylamino}ethyl]anthra[1,9-*c*,*d*]pyrazol-6(*2H*)-one (G) [70,71]

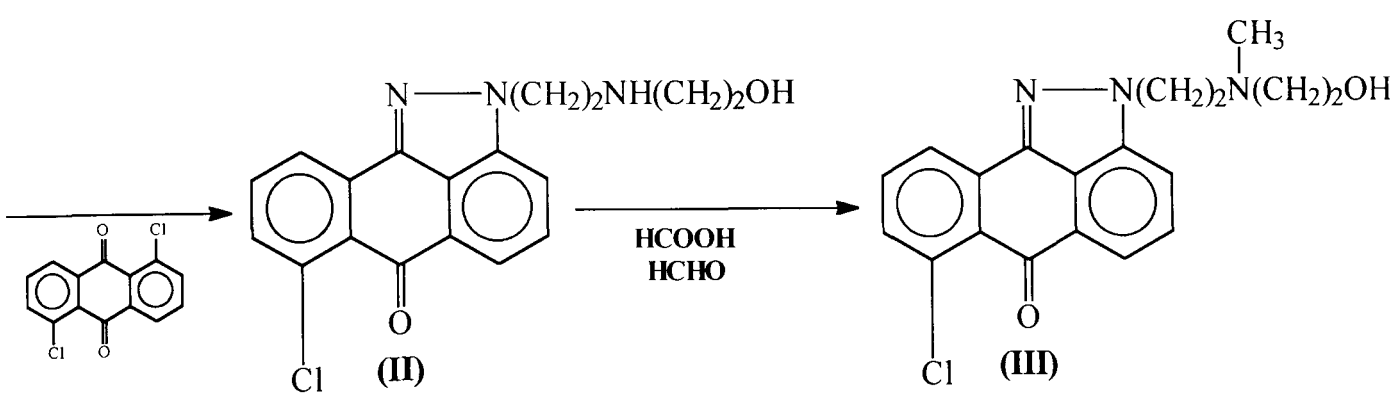
Product

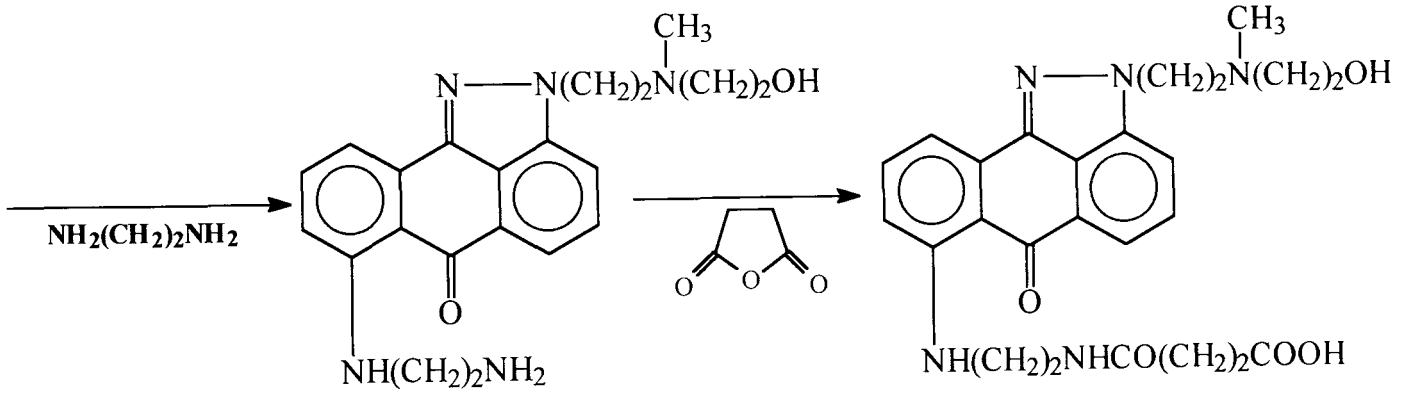


Intercalator-linker G was employed to assess the effect of a second side-chain on the DNA binding of the peptide moiety. Synthesis of this intercalator-linker involved five reactions, the first three of which were essentially the same as those described in the synthesis of intercalator-linker F, except that 1,5-dichloroanthraquinone was used in place of 1-chloroanthraquinone. Attachment of the second side-chain involved sequential amination and amidation with ethylenediamine and succinic anhydride respectively, as in the synthesis of intercalator-linker A (section 3.5.3.1). The product was isolated in an 11.5% overall yield.

Reaction Scheme 15

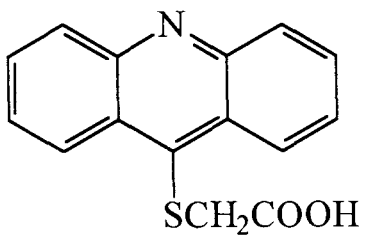






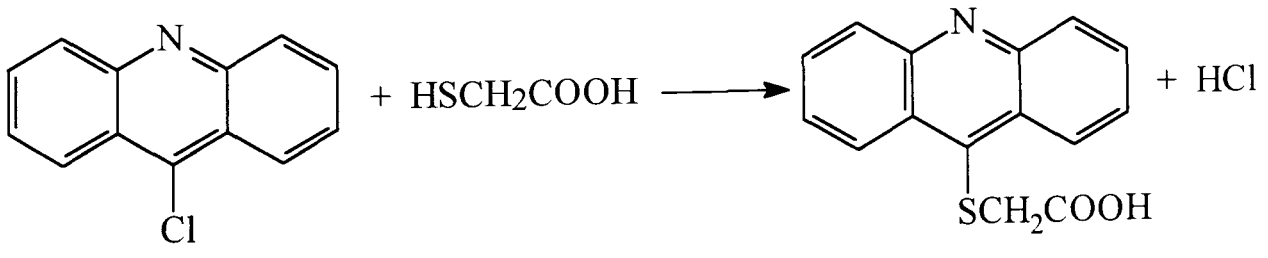
3.5.3.7 Synthesis of 9-[*S*-{Carboxymethyl}mercapto]acridine (H)

Product



The acridine chromophore, a known intercalator of DNA, was also investigated as an alternative to the anthraquinone intercalataor [32,53]. 9-Chloroacridine was reacted with thioglycolic acid in methanol in the presence of DIEA. Thioglycolic acid was used as opposed to an amino or amino acid moiety due to the stronger nucleophilic nature of the sulphur atom and its ability to avoid zwitterion formation. The product was isolated as yellow solid, in a 70% yield after column chromatography.

Reaction Scheme 16



3.5.4 Attempted Synthesis

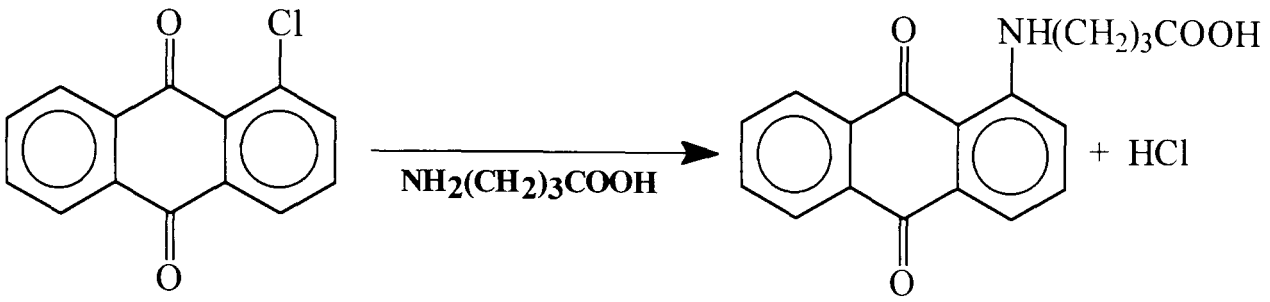
In addition to those described earlier a number of alternative intercalator-linkers were also investigated, the isolation of these however was unsuccessful. The next section briefly describes the attempted synthesis of these compounds.

2.5.4.1 Synthesis of 1-[*N*-{3-Carboxypropyl}amino]anthraquinone

Synthesis of 1-[*N*-{3-carboxypropyl}amino]anthraquinone involved a nucleophilic displacement reaction, in which the chloro group of 1-chloroanthraquinone was to have been displaced by the amino group of γ-aminobutyric acid (GABA). Unfortunately, although this reaction is facile from a mechanistic point of view, a number of problems were encountered in practice.

An initial attempt to synthesise 1-[*N*-{3-carboxypropyl}amino]anthraquinone in methanol with a few drops of acetic acid was unsuccessful, this was possibly due to the complete protonation of the amino group of GABA. Replacing acetic acid with triethylamine, in order to prevent zwitterion formation, also gave no product. Finally use of NaOH (1M) and the Lewis acid $ZnCl_2$ in methanol provided the desired product in a 50 % crude yield. Use of 2-methoxyethanol allowed the temperature to be raised from 65 to 125°C. Although this enhanced the reaction rate, it had no effect on the reaction yield.

Reaction Scheme 17

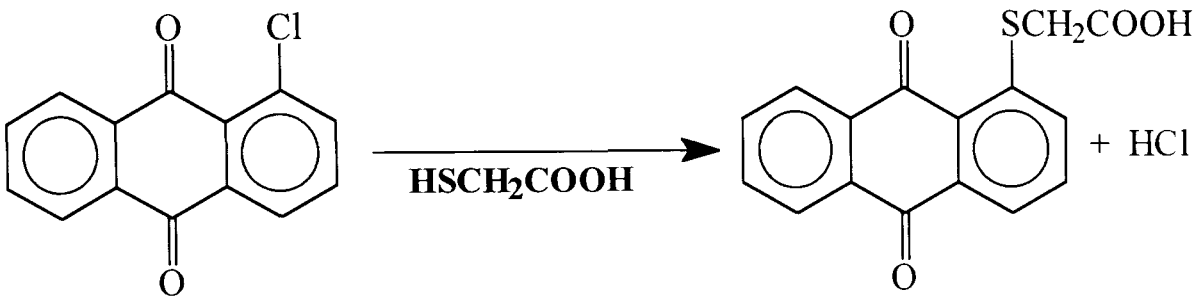


Purification of 1-[*N*-{3-carboxypropyl}amino]anthraquinone was extremely difficult, as the product was only sparingly soluble in all assessed polar protic, polar approtic and non-polar solvents. The problems encountered with the solubility were attributed to the large difference in polarity of the non-polar anthraquinone chromophore and polar GABA side-chain. A number of attempts were made in order to purify the product. Techniques such as recrystallisation, column chromatography, dry flash chromatography and preparative thin layer chromatography were employed, however these methods of purification were unsuccessful.

3.5.4.2 Synthesis of 1-[*S*-{Carboxymethyl}mercapto]anthraquinone

1-[*S*-{Carboxymethyl)}mercapto]anthraquinone was synthesised in the same manner as 1-[*N*-{3-carboxypropyl}amino]anthraquinone. Purification of this yellow product was unsuccessful as similar problems were encountered with the product's solubility as those indicated for 1-[N-{3-carboxypropyl}amino]-anthraquinone.

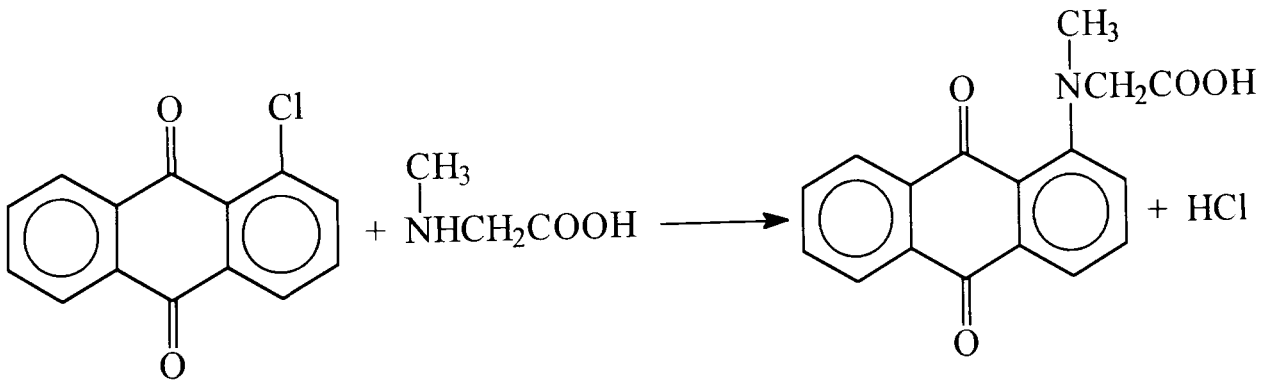
Reaction Scheme 18



3.5.4.3 Synthesis of 1-[*N*-Methyl-N-carboxmethyl]anthraquinone

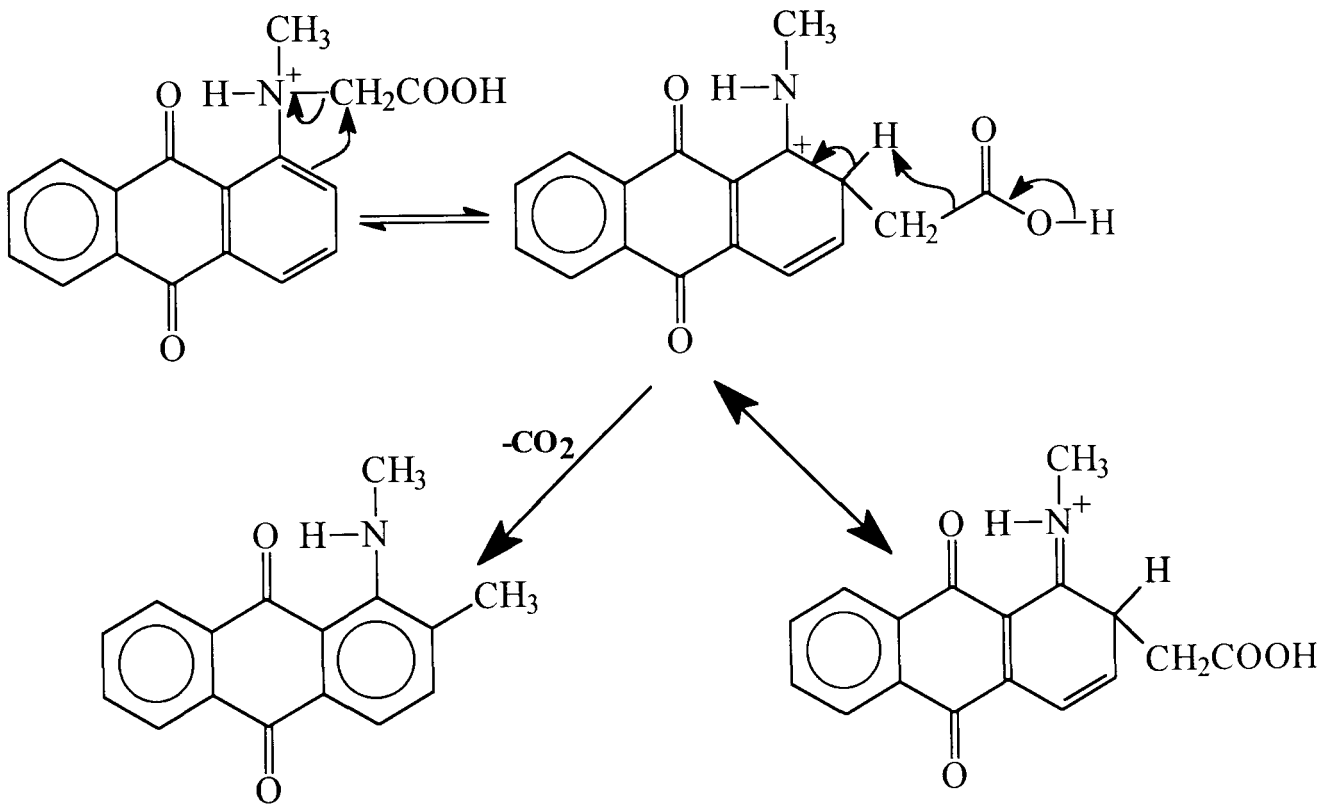
The synthesis of 1-[*N*-methyl-N-carboxmethyl]anthraquinone (reaction scheme 19) was attempted to determine the effect of a tertiary amino group on the solubility of the anthraquinone-amino acid derivative. Sarcosine and 1-chloroanthraquinone were refluxed in dimethylsulfoxide (DMSO) under nitrogen and a red product was isolated. This was purified using column chromatography from which two fractions were collected and analysed by ^{1}H-NMR.

Reaction Scheme 19



The NMR spectra indicated the presence of NCH_3 and $ArCH_3$ groupings but the resonance attributable to the expected NCH_2CO_2H was not observed. This finding might be explained if the desired product had been formed, but had subsequently undergone rearrangement and decarboxylation as shown below.

Reaction Scheme 19

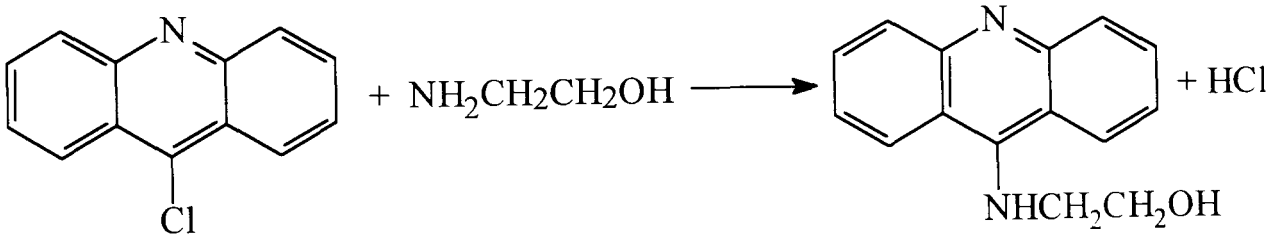


3.5.4.4 Synthesis of 9-[*N*-{2-Hydroxyethyl}amino]acridine

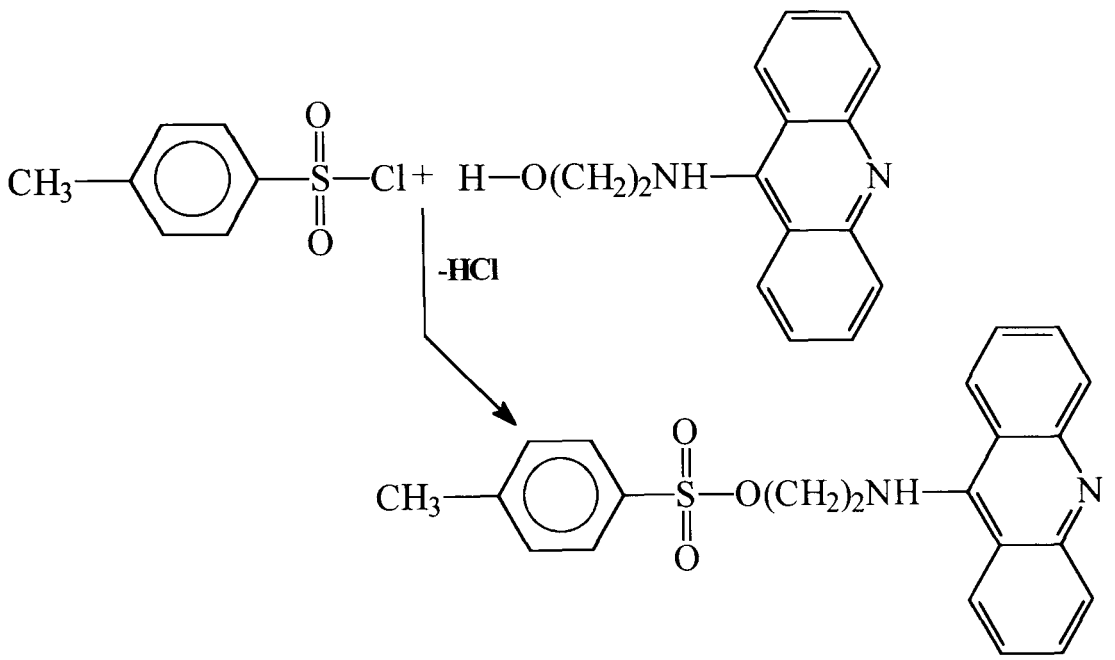
Synthesis of 9-[*N*-{2-hydroxyethyl}amino]acridine was achieved by reacting 9-chloroacridine with excess ethanolamine at room temperature. The product, after purification was then treated with *p*-toluenesulphonyl chloride in dry pyridine to afford its corresponding tosyl ester. Purification of this ester was attempted several times using column chromatography.

Reaction Scheme 20

I Amination



II Tosylation



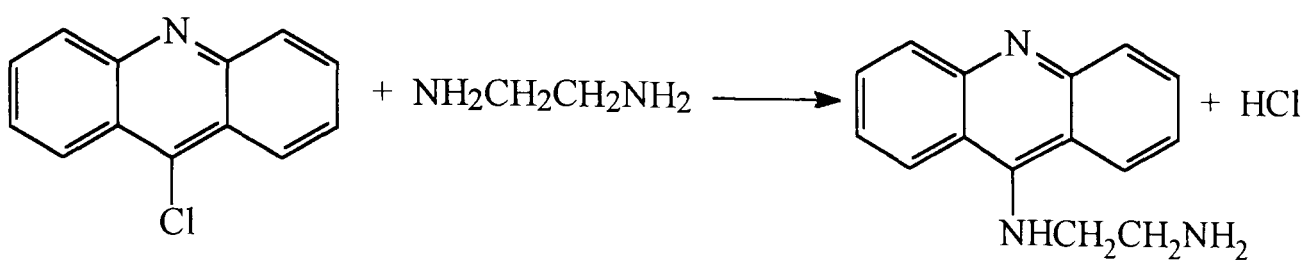
NMR of the final product however suggested that the tosyl ester had decomposed, regenerating the amino alcohol. Due to the problems encountered with the alkylation of the peptides with intercalator-linkers E and F, which became apparent in parallel studies, the synthesis of alkylating moieties was no longer pursued.

3.5.4.5 Synthesis of 9-[2-{Succinamylethyl}amino]acridine

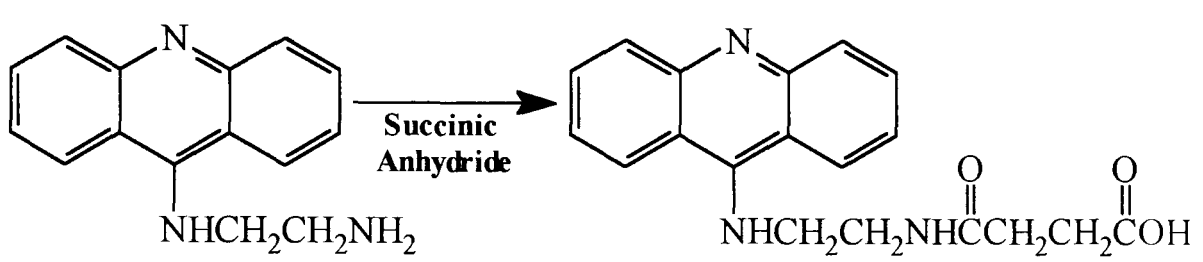
Synthesis of 9-[2-{succinamylethyl}amino]acridine was attempted using the method employed to synthesise compound A.

Reaction Scheme 21

I Amination



II Amidation



Isolation of the desired product was not achieved as amination of 9-chloroacridine resulted in dimer formation despite the use of excess diamine and dilute solutions (figure 30). This reaction was therefore no longer pursued.

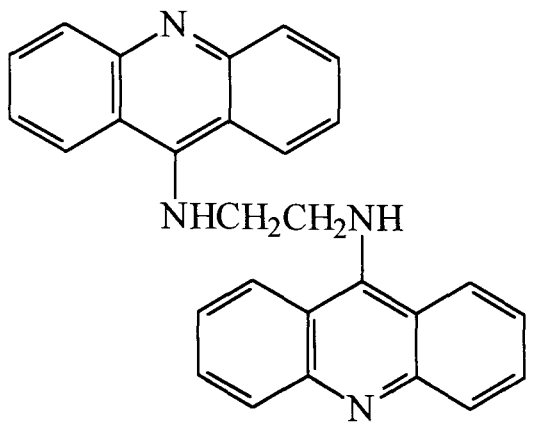


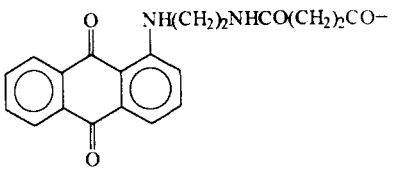
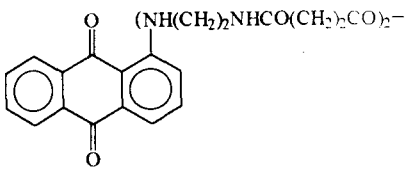
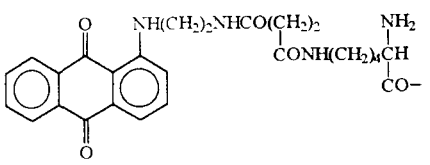
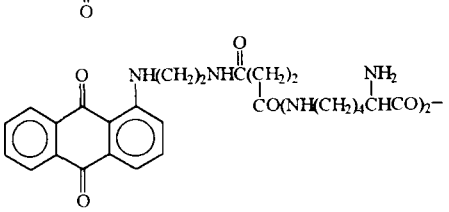
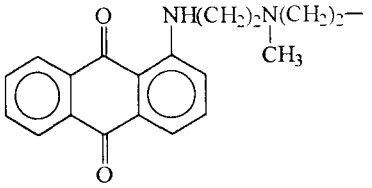
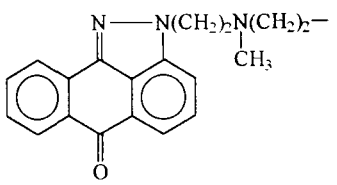
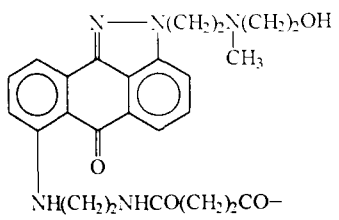
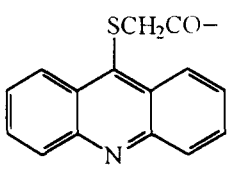
Figure 30 Acridine Dimer

The intercalator-linkers that were successfully synthesised were attached to the peptides described in section 3.4, figure 24. The next section describes the procedures involved in the preparation of the final intercalator linked peptides.

3.6 Peptide Conjugates

This section describes the methods employed in synthesising the peptide conjugates. The intercalator-linkers used and the conjugates synthesised have been shown in table 3.

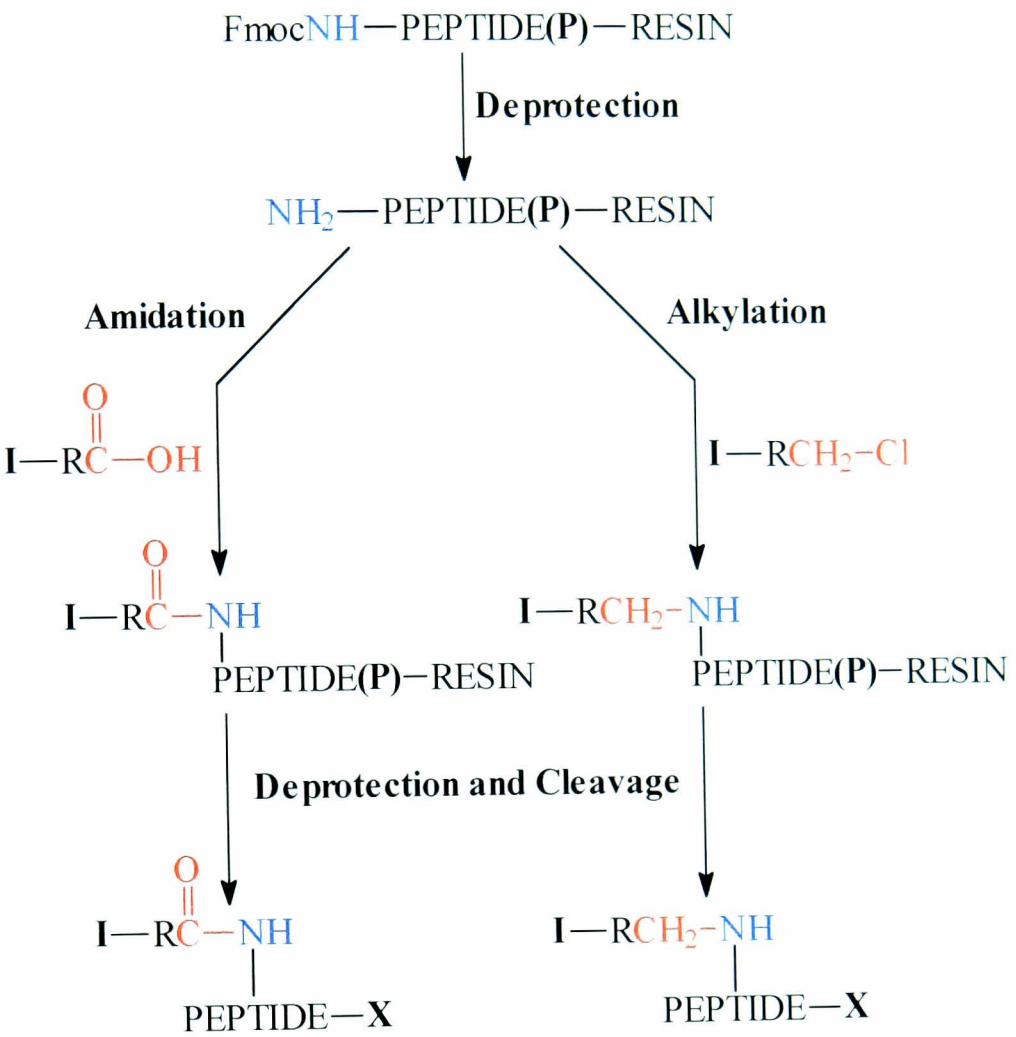
Table 3 Peptide Conjugates

Intercalator-linker		Peptide							
		1	2	3	4	5	6	7	8
	**A**	✓	✓	✓	✓	✓	✓	✓	✓
	**B**	–	–	–	✓	–	–	✓	–
	**C**	–	–	–	✓	✓	✓	✓	✓
	**D**	–	–	–	–	✓	✓	–	–
	**E**	✓	✓	✓	✓	–	–	–	–
	**F**	✓	✓	–	–	–	–	–	–
	**G**	–	–	–	✓	–	–	✓	–
	**H**	–	–	–	✓	–	–	–	–

✓ Attempted Synthesis: Peptides 1:ARCKA 2:AKCRA 3:AKSRA 4:AKCRNA 5:AKCRKA 6:AKCRNRA 7:AKCRKRA 8:AAKCRAA

3.6.1 Conjugate Synthesis

The N-terminal Fmoc group of each peptide was removed selectively with 20% piperidine in DMF. The free amino terminal of the peptide was then amidated or alkylated with the appropriate intercalator-linker whilst still attached to the solid support. Use of resin-supported peptide facilitated the use of excess alkylating/acylating components. Following extensive washing of the resin, isolation of the conjugates was achieved by treating the resin with a cocktail of scavengers in TFA as described in section 3.2.5. The protocols adopted for the carboxyl terminal intercalator-linker conjugates are discussed in section 3.6.2 and for the mustard conjugates in 3.6.3. The general protocol, however, is shown in figure 31.



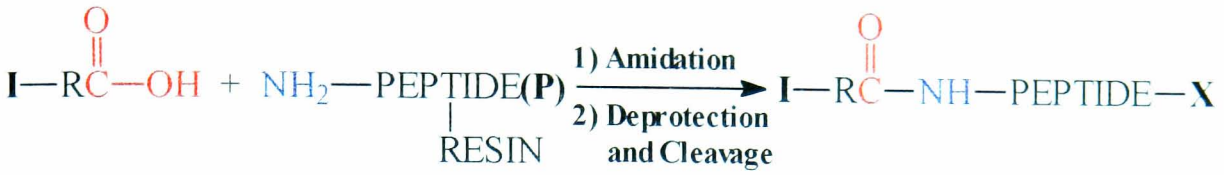
I =Intercalator; R = Linker; PEPTIDE(P) = Protected Peptide; X = COOH or $CONH_2$ terminus

Figure 31 Protocol Adopted for Conjugate Synthesis

3.6.1.1 Synthesis of the A-D, G and H Conjugates

Intercalator-linkers bearing a carboxyl terminus were amidated with the free amino terminal of the appropriate peptide for 3 hours using PyBOP and DIEA in DMF.

Reaction Scheme 22



I = Intercalator; R = Linker; PEPTIDE(P) = Protected Peptide; X = COOH or $CONH_2$

The conjugate bound resin was washed sequentially with DMF, ethanol and ether to remove the excess of reagents and any impurities present, after which the product was cleaved from resin. Analysis of the conjugates using MALDI-MS suggested that all the desired products except 4H had been isolated, figure 32 shows a typical spectrum. The peak at 907 daltons corresponds to the desired peptide conjugate (1A), the peak at 365 is a component of the MALDI matrix. The spectrum of 4H indicated the presence of two components with molecular weights of 705 and 1409, neither of which corresponded to the free peptide or the desired product. The identity of the two products was not resolved. Whilst the higher molecular weigh impurities might suggest the presence of the partially protected peptide, there is no obvious explanation as to why the conditions utilised should fail in achieving complete deprotection in this case alone.

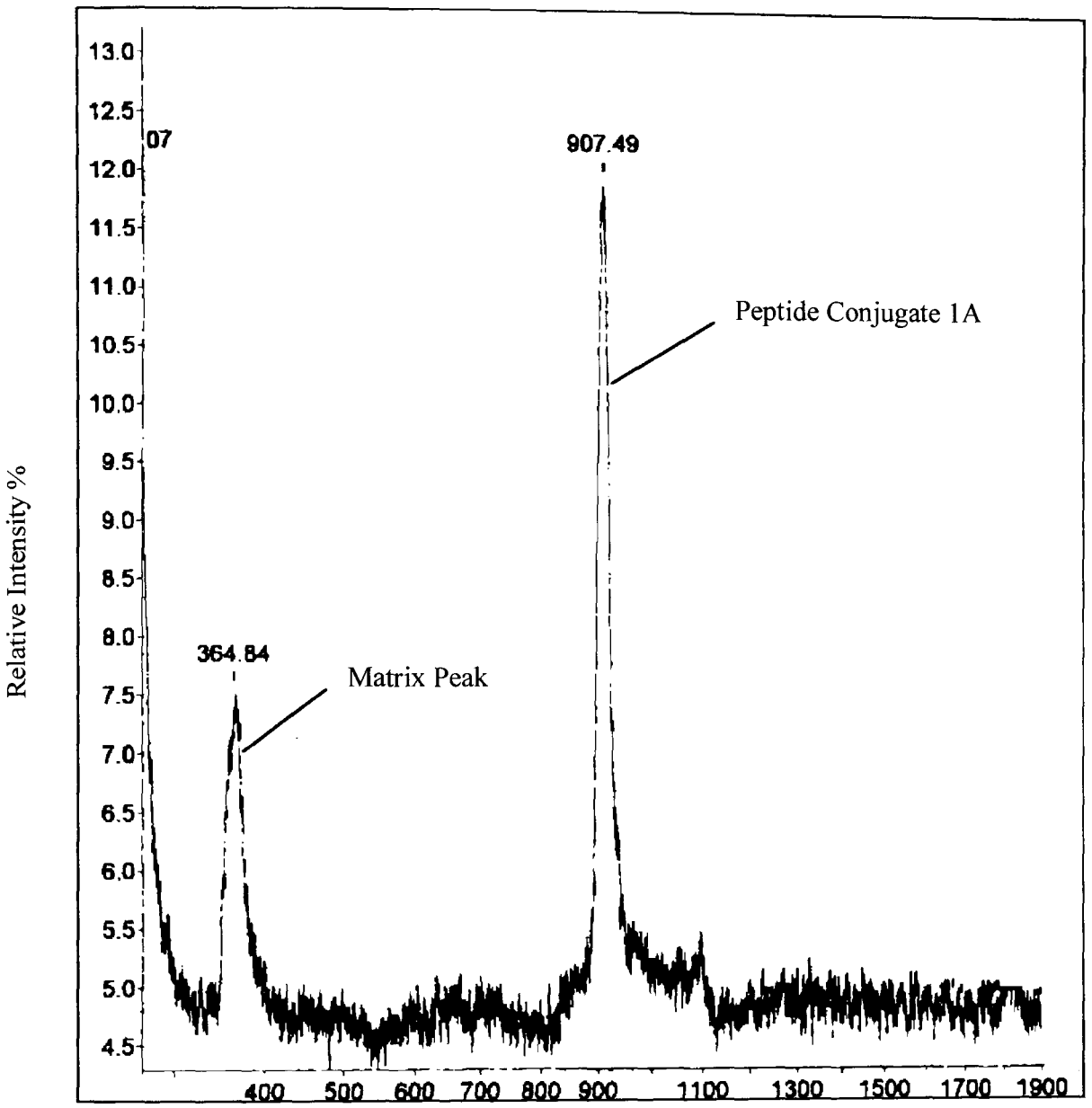
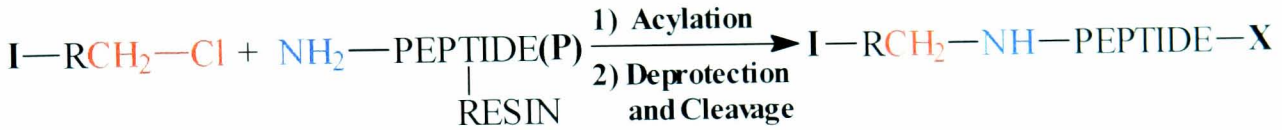


Figure 32 MALDI Mass Spectrum of Peptide Conjugate 1A (refer to table 3 for conjugate structure)

3.6.1.2 E and F Conjugates

The E and F conjugates were synthesised by alkylating the resin bound peptides with the appropriate intercalator-mustard. Peptides 1 to 4 and 1 to 2 were alkylated with intercalators B and C respectively in DMF, in the presence of DIEA for 3 hours.

Reaction Scheme 23



I = Intercalator; R = Linker; PEPTIDE(P) = Protected Peptide; X = COOH or $CONH_2$

Analysis of these conjugates by MALDI-MS, however indicated that a pure form of the desired products had not been isolated. The spectra suggested that the alkylation of the peptide in each case was incomplete. This was indicated by the presence of a major and minor peak, which correlated to the free peptide and its corresponding conjugate respectively, as shown in figure 33.

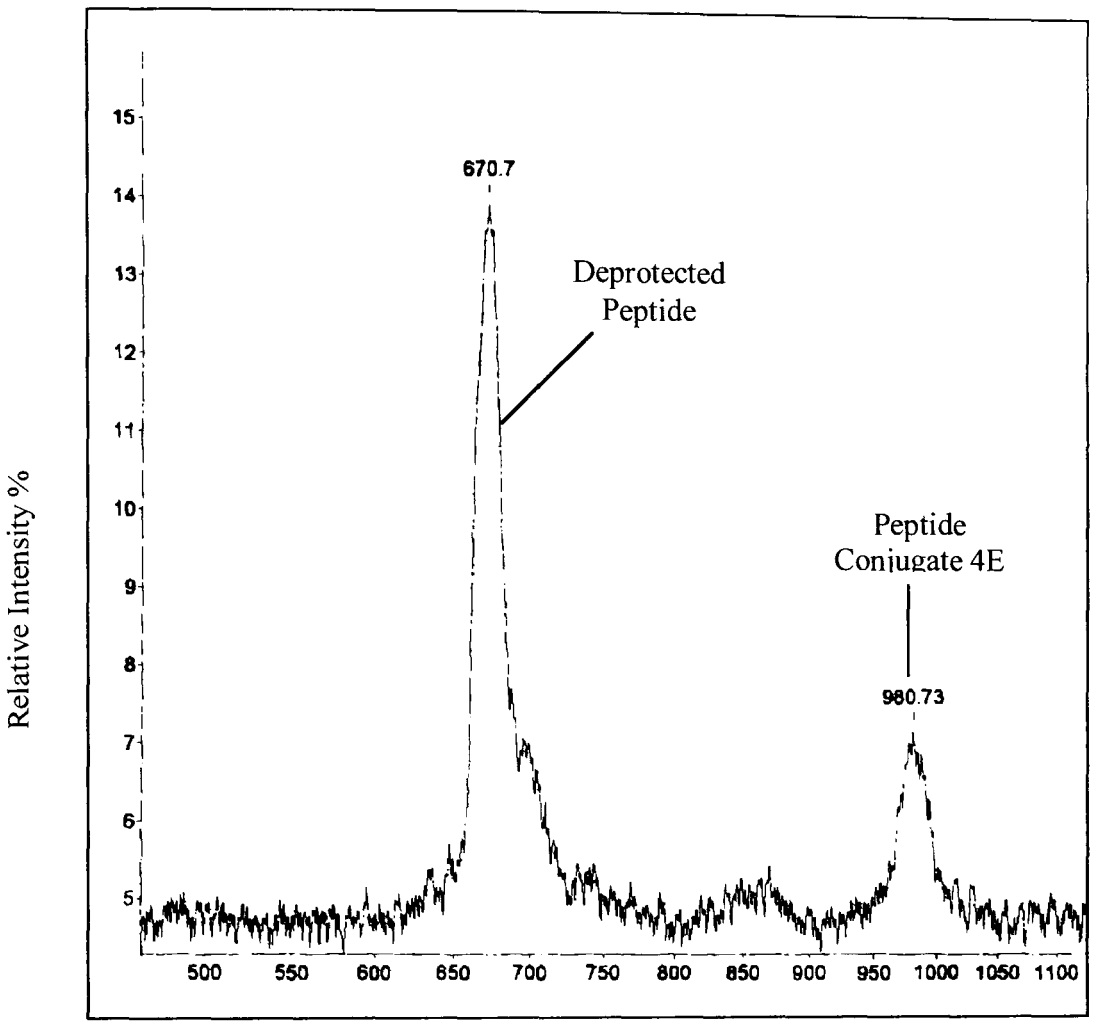


Figure 33 MALDI Spectrum of Peptide conjugate 4E (refer to table 3 for conjugate structure)

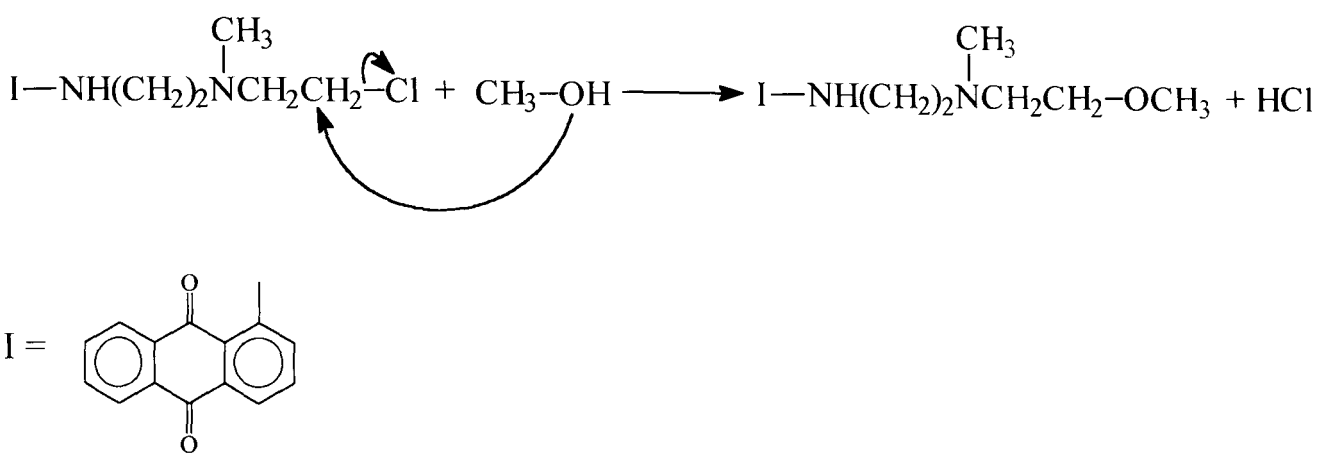
Preparation of 4E was attempted several times using various reaction conditions. It was anticipated that repeated alkylation of the peptide with E would ensure that a 100% reaction would be achieved. A small aliquot of peptide 4 (resin bound) was alkylated twice and a further aliquot was alkylated four times with the E intercalator-linker, after which the products were cleaved from resin. Analysis of 4E in both cases using MALDI-MS showed that there was no marked improvement in the alkylation of peptide 4. An alternative base to DIEA, i.e. N, N-dimethylaminopyridine (DMAP) was also employed in the anticipation that this would enhance the reaction yield. However the MALDI mass spectrum of the product isolated indicated that no improvement in the reaction had occurred. In a final attempt to alkylate the peptide, the reactions were conducted at 30°C and at 45°C in the presence of DIEA. Although the reactions were left for up to 18 hours complete alkylation was not achieved. The poor alkylation of the peptides with both E and F suggested that these compounds were either undergoing degradation or that a portion of the resin-bound peptide had aggregated in such a fashion that it was no longer nucleophilic.

3.6.1.2.1 Reactivity Studies

Stability studies were conducted in an attempt to establish whether the intercalator-linked mustards were undergoing degradation. The stability of E was determined in both DMF and methanol, both in the presence and absence of DIEA. The experiments were conducted at room temperature and at 45°C, and were monitored using TLC at regular time periods for 18hours. Degradation of E was anticipated to

be more pronounced in methanol, in the presence of base, as it was expected it to undergo nucleophilic attack by the solvent to afford its methoxy derivative as shown in reaction scheme 25.

Reaction Scheme 24



The results obtained from these experiments indicated that E was relatively stable, although its methoxy derivative was observed in methanol when heated at 45°C in the presence of DIEA. This however was only present in a minute quantity after 18 hours. These results therefore indicate that the earlier lack of success in alkylation of the peptide was not due to the instability of the intercalator-linkers but rather to their poor reactivity.

3.6.1.2.2 Model Alkylation Studies

The reactivity of E using benzylamine as a model was investigated in the anticipation that this would help establish the conditions necessary for the complete and successful alkylation of the peptides. Benzylamine was reacted with B under several conditions; these along with the results obtained are summarised in table 4. All reactions were monitored at regular time periods using TLC.

Reaction Scheme 25

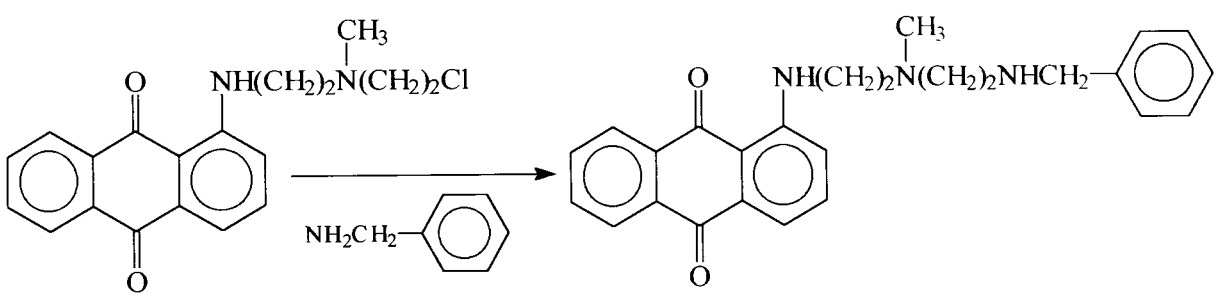


Table 4 Reactivity Studies of Intercalator-linker E using Benzylamine

	Molar Equivalents of Reagents					
Method	**Inercalator-Linker E**	**Benzylamine**	**Base**	**Solvent**	**Temperature (°C)**	**Results**
A	**4**	**1**	**DIEA**	**DMF**	**25**	**No significant reaction after 70 hours**
B	**4**	**1**	**DIEA**	**DMF**	**37**	**Complete reaction after 76 hours**
C	**4**	**1**	**—**	**DMF**	**37**	**Complete reaction after 100 hours**
D	**1**	**Excess**	**—**	**—**	**>60**	**Complete reaction after 5 minutes**

The results in general indicate that the alkylation of benzylamine with E is very much dependent on three factors, i.e. concentration, time and temperature. If the reaction is allowed to proceed at 37°C for a significant length of time then complete alkylation may be achieved, although if heated vigorously in excess amine the desired product will form almost instantaneously. Unfortunately, these extreme conditions cannot be employed in the synthesis of the desired conjugates, as the peptides are likely to undergo racemization and degradation. For this reason the synthesis of the E and F conjugates was no longer pursued.

3.7 An Alpha Helical Constraint

In addition to preparing unrestrained peptides in this project, the synthesis of a helical constrained peptide was also attempted. The method adopted was that described by Phelan and co-workers [50] in which an amide tether is formed between the *i* and *i*+7 residues of a peptide. In this case 1,4-diaminobutane was employed to bridge the glutamic acid residues at positions *i* and *i*+7 in a peptide, (i.e. peptide 9), bearing the KCR motif, figure 34. This section briefly describes the procedures and reagents employed in an attempt to constrain peptide 9 in a helical conformation.

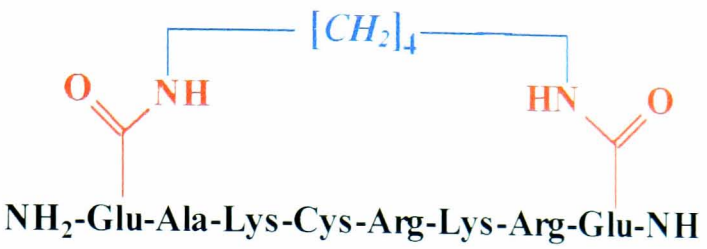


Figure 34 Peptide Nine Constrained using an Alkanediamine Chain

It was expected that this conformational constraint would preferentially lock the peptide in an α-helical structure which is reminiscent of the basic domains of the AP-1 protein when bound to DNA. It was anticipated that this amide bridge or constraint would prevent random coiling of the peptide and hence possibly increase the affinity of the peptide for the DNA AP-1 site. This is highly plausible since it has been previously reported that upon recognition of the DNA AP-1 consensus sequence the basic regions of the fos and jun proteins become completely helical, thus allowing tight binding interactions [21]. The potential increase in affinity of the peptide for DNA would be expected as a result of a favourable change in the entropy of the binding to DNA. It was expected that there would be a relatively small loss of entropy upon binding of the constrained peptide to the DNA AP-1 site as it would already be in its binding conformation. This is unlike the binding of the unrestrained peptides which, due to a loss of conformational flexibility, results in a considerable loss of entropy. It was therefore anticipated that DNA binding of the constrained peptide, i.e. peptide 9 will occur more readily compared to that of the unrestrained peptides.

3.7.1 Peptide 9

Peptide 9 was synthesised by Calbiochem-Novabiochem Ltd using SPPS and was supplied fully protected and bound to resin. The amino terminus was protected with the Fmoc group and all of the side chain functionalities, except those of the Glu residues were masked with various acid labile protecting groups. The carboxyl functions of the glutamic acid (Glu) residues at positions *i* and *i* + 7 were protected with ODmab and the hyper-acid labile group Opfp respectively. ODmab can be removed selectively using 2% hydrazine in DMF and Opfp is deprotected by 1% TFA in dichloromethane.

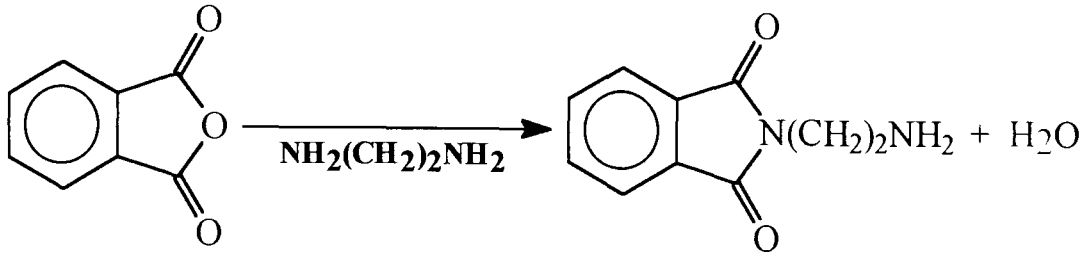
3.7.2 Amide Bridge

1,4-Diaminobutane, which affords a 4-methylene bridge, had been previously shown to provide an optimal length for inducing and sustaining α-helicity in short peptides, thus preventing randomisation of the secondary structure [50]. Monoprotection of this diamine prior to its attachment to the peptide was necessary to prevent any undesirable reactions. It was prerequisite that the masking group used to monoprotect 1,4-diaminobutane could be removed selectively and was stable to the conditions required to deprotect the two glutamic acid residues.

Phthalic anhydride, which can be removed using 24% hydrazine in methanol [74] was employed to monoprotect the 1,4-diaminobutane. The desired product was synthesised by refluxing this diamine with phthalic anhydride in chloroform for 5

hours [74]. A white precipitate was isolated and purified using column chromatography to give the product in a 20% yield.

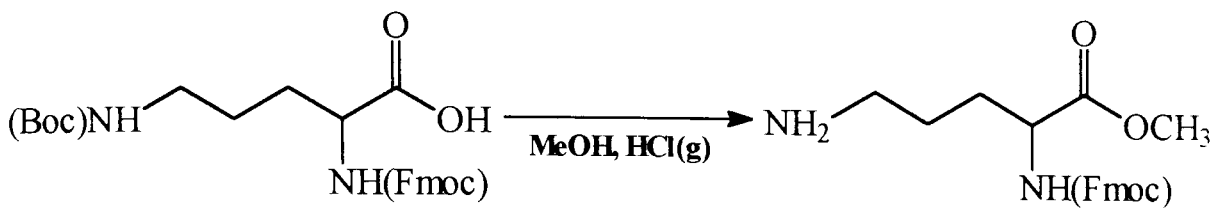
Reaction Scheme 26



3.7.2.1 An Alternative Linker

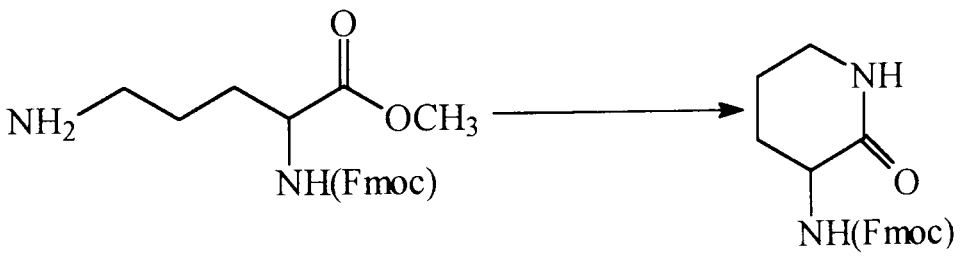
The amino acid ornithine, which also provides a 4-methylene bridge, was employed as an alternative to 1,4-diaminobutane. This residue was obtained from Calbiochem-Novabiochem Ltd in the protected form of Fmoc-Orn(Boc)OH. In order to attach this residue to peptide 9, it was first necessary to remove one of its two protecting groups and also to mask its carboxyl terminus. The latter was protected with a methyl ester function under acidic conditions, which therefore resulted in the removal of the Boc protecting group. Fmoc-Orn(Boc)OH was dissolved in methanol and saturated with dry hydrogen chloride gas, which resulted in the simultaneous protection and deprotection of the carboxyl and amino functions respectively. The product was isolated as an off-white solid, which was purified using column chromatography.

Reaction Scheme 27



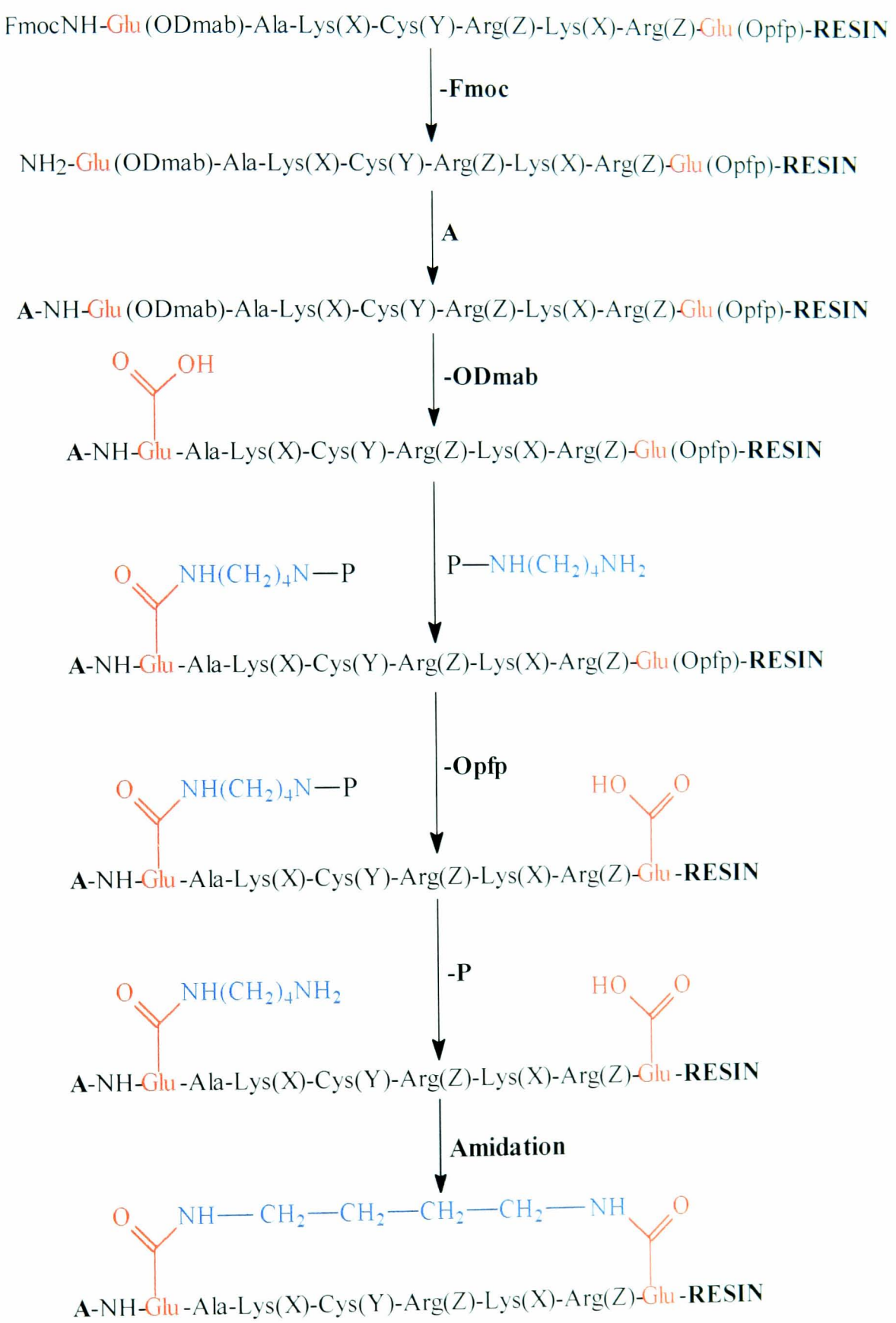
NMR analysis of the product however suggested that despite the presence of excess acid, it had undergone cyclisation as shown in reaction scheme 28. This unexpected lactam formation, coupled with the difficulties in completing the 4-methylene bridge (discussed later in 3.7.3) led to the discontinuation of this synthesis.

Reaction Scheme 28



3.7.3 Synthesis of a Constrained Peptide

The general scheme adopted for constraining peptide 9 using 1,4-diaminobutane is summarised in figure 35. Prior to each reaction involving acylation, the resin was solvated with DMF and at the end of each reaction the resin was washed thoroughly with DMF, ethanol and ether.



A, Intercalator-linker A; P, Phthalyl; X, Boc; Y, Trt; Z, Pbf

Figure 35 Protocol Adopted in Constraining Peptide Conjugate 9A

In the first step of the protocol the N-terminal was selectively deprotected and acylated with intercalator-linker A to afford 9A using the conditions described previously in section 3.5.3.1. The carboxyl function of the Glu residue at position *i* was deprotected and amidated with the phthalimide protected diamine linker using PyBOP. Removal of the phthalimide group was achieved using 24% hydrazine in methanol. The second Glu residue was deprotected and amidation was attempted with the free amino entity of the 1,4-diaminobutane bridge. The peptide conjugate (figure 36) was fully deprotected and cleaved from resin using TFA as described in section 3.2.5.

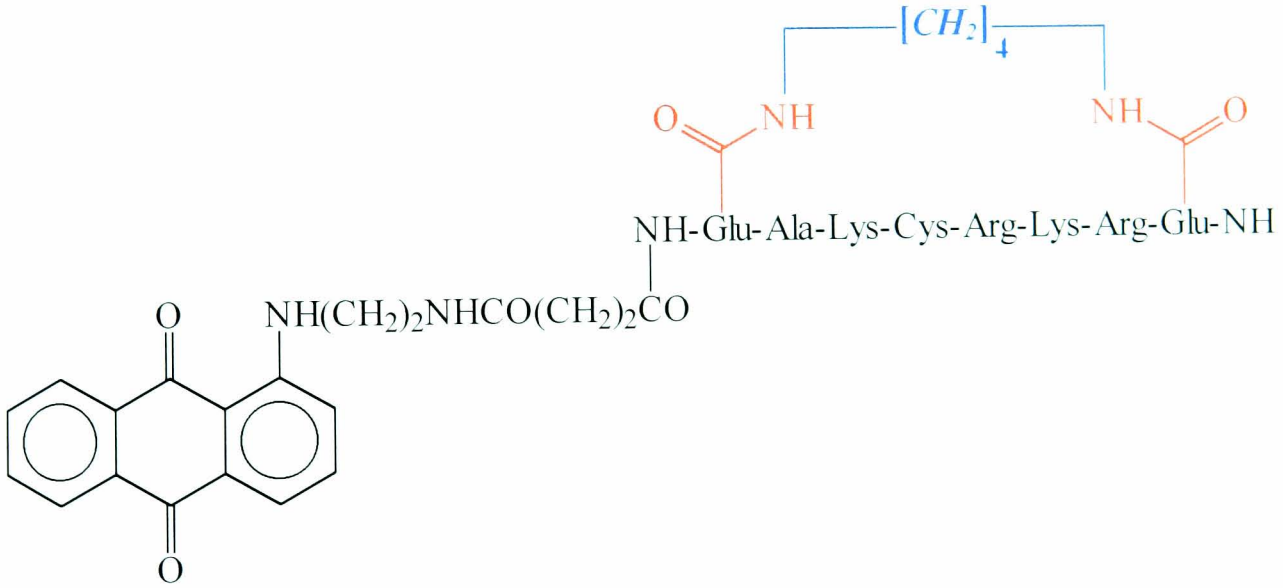


Figure 36 Peptide Conjugate 9A

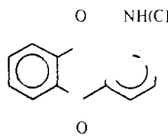
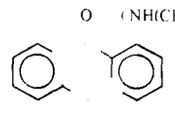
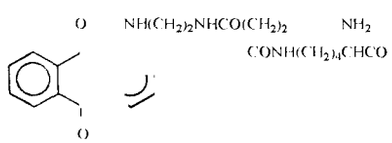
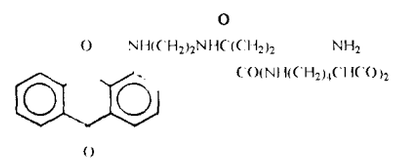
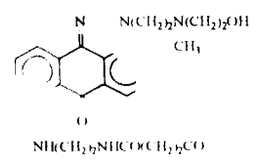
Analysis of peptide conjugate 9A using MALDI-MS, however indicated that the desired product had not been isolated. There are two possible explanations for the failure of this particular synthesis, i.e. peptide chain aggregation and steric

hindrance. The former arises from either hydrophobic interactions or interchain hydrogen bonding and often results in incomplete coupling and deprotection. This is however is unlikely as acylation of peptides 1-8 occurred satisfactorily. Steric hindrance is created by the bulky nature of the side-chain functionalities of the amino acid residues and their respective protecting groups. It is highly probable that the large Pbf protecting group used to mask the Arg residue (at position *i* +*6*), the Trt Cys protection and the Boc protecting group of the Lys residue prevented the coupling of the Glu residue to the free amino group of the butyl bridge. If indeed these groups are preventing closure of the constraining butyl bridge, it is likely that the bridge would mask the critical KCR functional moiety of the peptide were closure achieved and hence disrupt DNA binding of the peptide. For these reasons this approach was no longer pursued.

4 EVALUATION – RESULTS AND DISCUSSION

The peptides and final conjugates synthesised were evaluated both biologically and structurally using the techniques described in chapter 2. For convenience, table 5 provides a summary of the compounds evaluated.

Table 5 The Peptide Conjugates Synthesised and Evaluated Biologically and Structurally

Intercalator-Linker		Peptide Sequence							
		1 ARCKA	2 AKCRA	3 AKSRA	4 AKCRNA	5 AKCRKA	6 AKCRNRA	7 AKCRKRA	8 AAKCRAA
	**A**	✓	✓	✓	✓	✓	✓	✓	✓
	**B**	–	–	–	✓	–	–	✓	–
	**C**	–	–	–	✓	✓	✓	✓	✓
	**D**	–	–	–	–	✓	✓	–	–
	**G**	–	–	–	✓	–	–	✓	–

✓ Synthesised A = Ala C = Cys K = Lys N = Asn R = Arg S = Ser

4.1 Biological Evaluation

Spectrophotometric and fluorescence techniques as described in section 2.2 were employed to determine the nature of binding and the affinity of the peptide conjugates, shown in table 5, for calf thymus DNA.

4.1.1 Spectroscopic Studies

The spectral changes in the absorption characteristics of each conjugate upon binding DNA were measured and the spectra of solutions containing a fixed concentration of peptide conjugate and varying concentrations of DNA were superimposed to determine the presence of an isosbestic point, which is indicative of a DNA-binding interaction [52]. As discussed earlier in 2.2.2 the appearance of an isosbestic point occurs only where there is a single spectroscopically distinct bound form of the drug molecule in addition to the free drug (in solution) and where the spectra of free and bound drug superimpose. None of the conjugates tested in this study showed an isosbestic point, for example figure 37 shows the absorbance spectrum of peptide conjugate 1A. This meant that the binding affinity of these drugs could not be determined using the method of Scatchard in which the appearance of an isosbestic point is mandatory.

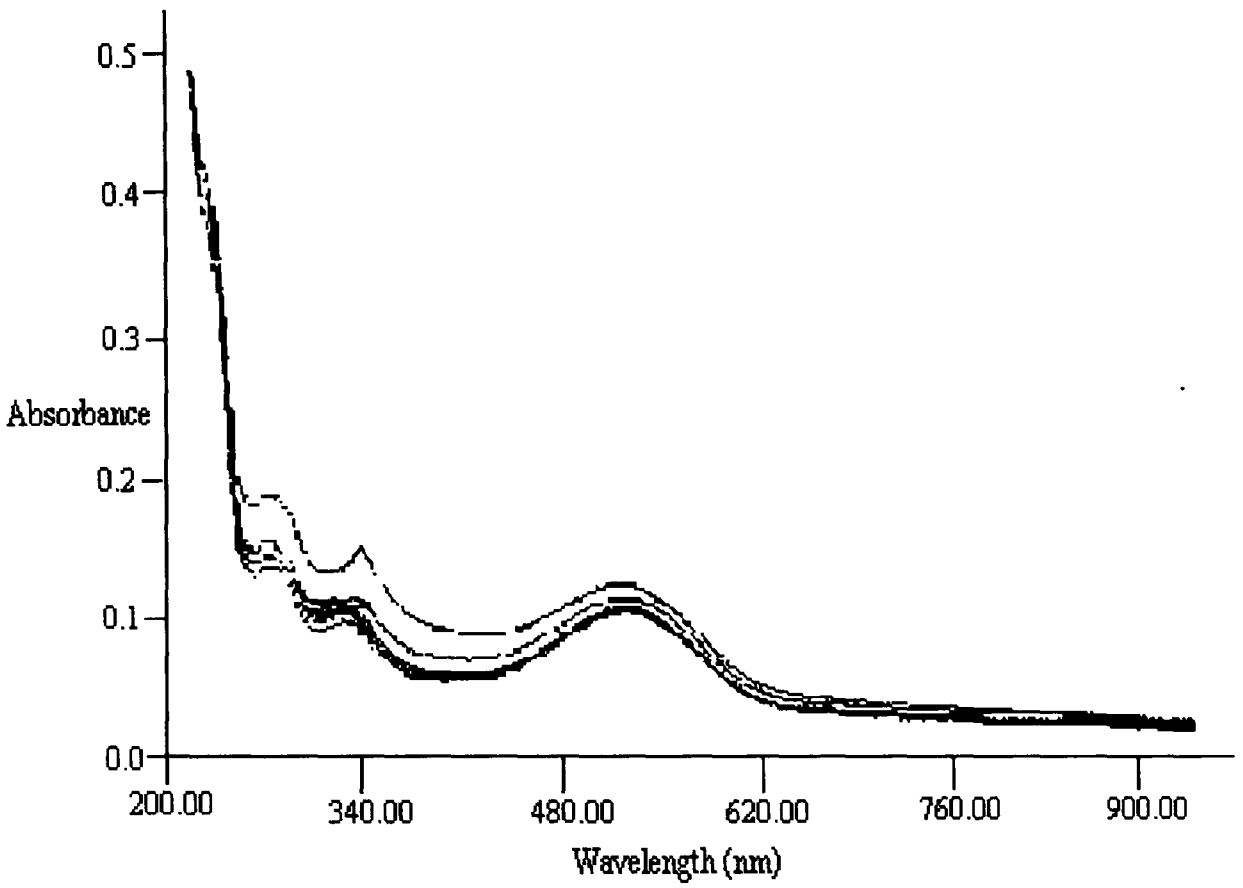


Figure 37 An UV-VIS Absorbance Spectrum to show the Effect of DNA on the Spectral Properties of Peptide Conjugate 1A (see 6.4.1 for experimental conditions)

The lack of spectral shifts and negligible decrease in extinction coefficient on interaction of the conjugates with calf thymus DNA could suggest that these drugs were not intercalating. However, an isosbestic point as a result of drug-DNA intercalation will not be observed if the spectra of free and bound forms of the drug do not cross when superimposed. Indeed, it has been previously shown that some drugs, for example proflavine, which are known to intercalate into DNA [75] do not show an isosbestic point [34]. An isosbestic point is only indicative of a single spectroscopically distinct bound form of the drug and not a requirement of intercalation. Hence the absence of an isosbestic point in this study does not necessarily eliminate intercalation as a mode of binding.

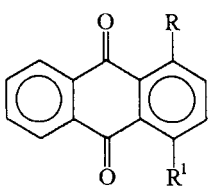
The results, however, do suggest that intercalation is not the only mode of binding exhibited by the conjugates and that it is possible that a second interaction between the conjugates and DNA may also be occurring. This mode of binding, known as 'external binding', is due to an electrostatic interaction between the cationic drug molecules and the polyanionic DNA sugar-phosphate chains [32]. External binding of the conjugates is highly plausible as the peptidic moiety of these drugs is very basic and therefore electrostatically compatible with the backbone of DNA. It is important to note that both intercalation and external binding of drugs upon complexation with DNA are dependent on ionic strength [32,52]. External binding can be eliminated experimentally by increasing the concentration of salt and hence ionic strength. Since the design principle of these conjugates requires a mixed mode of binding, that is, interaction of the

chromophore and surface binding of the peptide, inhibition of external binding was not desirable. For this reason, low salt concentrations (low ionic strengths) were used in these investigations.

The lack of intercalation observed with the conjugates is actually desirable as it suggests that the DNA binding affinity of the peptide is greater than that of the intercalating chromophore and that it is the peptidic moiety that governs DNA binding of the conjugate and hence potentially aids selectivity. This is further supported by the studies conducted by Gandecha [57], in which intercalators 1-[2-(hydroxyethylamino)ethylamino]anthracene-9,10-dione (I) and ametantrone (III) (see table 6) were shown to bind DNA, through intercalation, with a high affinity (1.03×10^6 M^{-1} and 2.8×10^6 M^{-1} respectively). Since the intercalator-linkers synthesised in this study are similar to those described in table 6, it can be assumed that as separate entities these moieties are likely to bind DNA in a similar manner, however upon attachment of a peptide fragment DNA binding of the chromophore is governed by the latter. It is also plausible that the lack of intercalation observed with the conjugates may also be due to the substitution pattern of the intercalating chromophore, as it has previously been established that mono-substituted anthraquinones bind DNA with a lower affinity compared to their bis-substituted analogues [57,76,77]. This is also apparent from the binding affinities of the intercalator-linkers described previously and shown in table 6.

It is, however, possible that the intercalative binding of the conjugates is considerably diminished where the peptide moiety stabilises the double helix, through an electrostatic interaction, by binding DNA in a way that prevents the unwinding of the double helix and hence intercalation of the drug. Although this form of interaction with DNA is favourable in that it stabilises the duplex and hence prevents transcription, it is problematical in that it shows no selectivity for the AP-1 site.

Table 6 DNA Binding Properties of Mono- and Bis-Substituted Anthraquinones [57,76]



	R	R^1	*Binding Affinity Constant ($K\ M^{-1}$)	**ΔT_m (°C)
I	$NH(CH_2)_2NH(CH_2)_2OH$	H	1.03×10^6	8.0
II	$NH(CH_2)_2N(CH_2CH_3)_2$	H	1.48×10^6	8.8
III	$NH(CH_2)_2NH(CH_2)_2OH$	$R^1 = R$	2.83×10^6	19.5

*A constant determined by spectrophotometric titration of drug with calf thymus DNA; ** The difference between the melting temperature of calf thymus DNA in the presence and absence of drug at 10:1 DNA-drug ratio.

4.1.2 Fluorescence Studies

Fluorescence enhancement studies using ethidium bromide as a fluorescent probe [34,55,56] were conducted to characterise the interaction between the peptide hybrids and calf thymus DNA. Conjugates of peptides 4 and 7 were tested using this form of spectroscopy since they exhibited the greatest activity i.e. ability to inhibit the AP-1 protein binding to its DNA consensus sequence (described later in section 4.1.4). Displacement of ethidium bromide from calf thymus DNA by the peptide conjugates 4A, 4B, 4C, 4G, 7A, 7B, 7C and 7G was investigated as described by Gandecha [57]. For each peptide conjugate a plot of fluorescence intensity against ethidium bromide:DNA ratio was constructed and in each case the fluorescence enhancement of ethidium was compared with that obtained for DNA in the absence of the drug.

The fluorescence enhancement of ethidium bromide was diminished with all the conjugates. Of the eight conjugates tested, 7C, 4G and in particular 7A showed the maximal displacement of ethidium bromide whereas 4A and 4C exhibited the least effect. Figures 38, 39 and 40 show the effects of peptide conjugates 4C, 7A and 7C on the fluorescence of ethidium bromide bound to DNA respectively and table 7 shows the percentage of fluorescence quenching (maximum) obtained with all the peptide conjugates analysed. The displacement of intercalated ethidium bromide from DNA by the peptide conjugates indicates that, to some extent, these drugs are binding to the same intercalation site. However the small decrease observed in the fluorescent enhancement of ethidium bromide with the conjugates

suggests that these agents are not very effective in competing for the DNA ethidium binding sites, either through lack of intercalation or through differing sequence selectivities. Since ethidium shows a preference for GC binding sites [32,34], it is possible that the peptide conjugates, like amsacrine [78] and some 1,5-substituted anthraquinones [76,77] are AT specific.

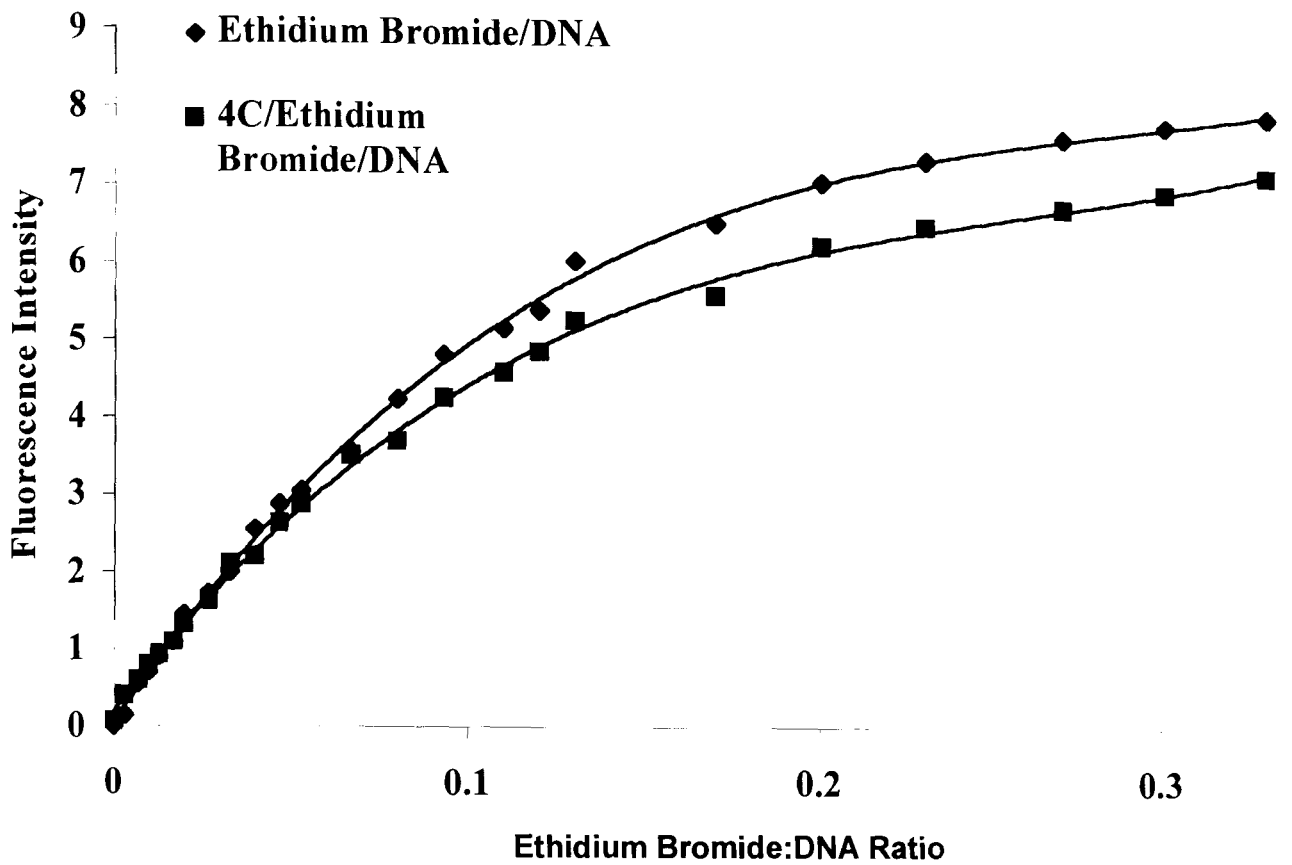


Figure 38 Effect of Peptide Conjugate 4C on the Fluorescent Enhancement of Ethidium Bromide Bound to DNA (see 6.4.2 for experimental conditions)

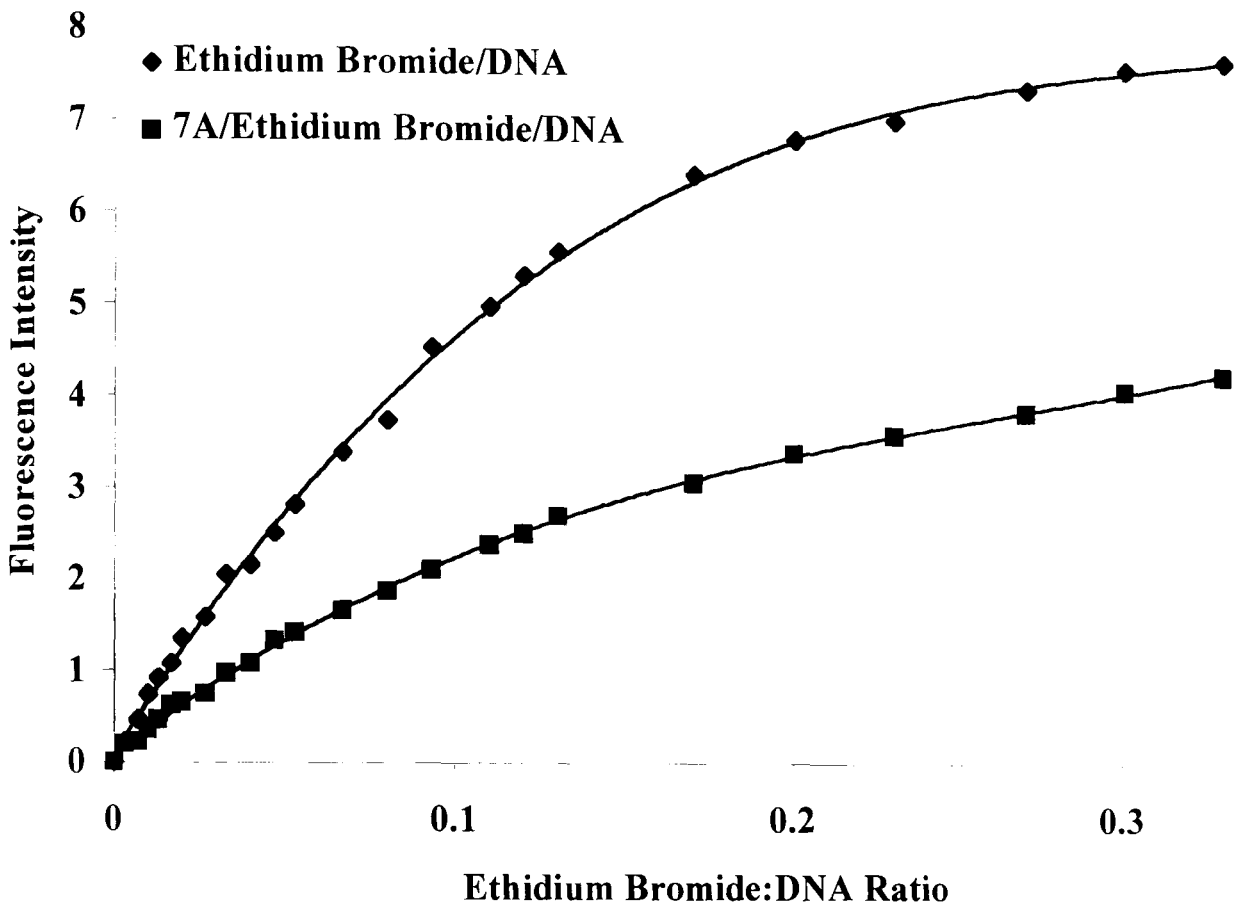


Figure 39 Effect of Peptide Conjugate 7A on the Fluorescent Enhancement of Ethidium Bromide Bound to DNA (see 6.4.2 for experimental conditions)

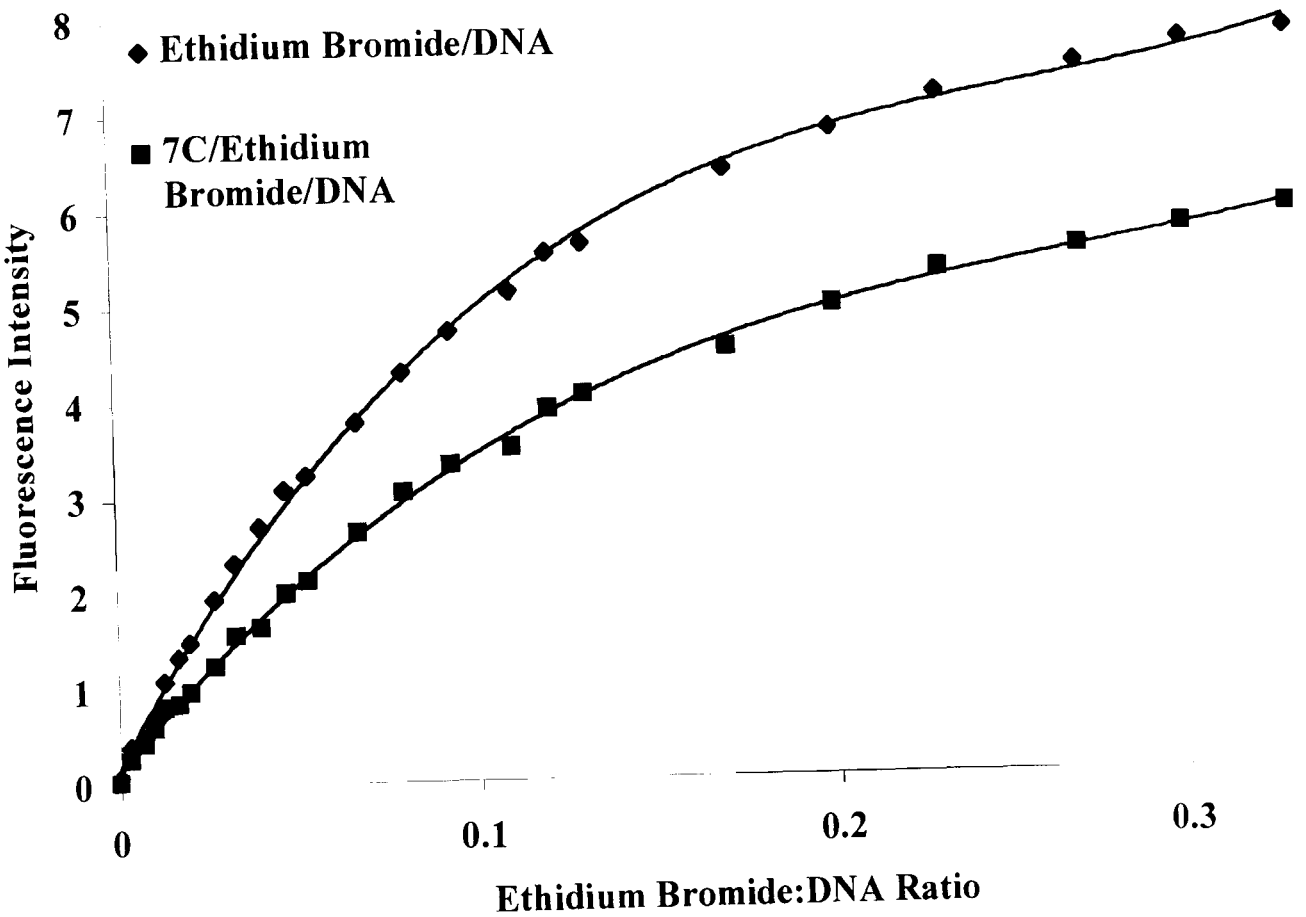


Figure 40 Effect of Peptide Conjugate 7C on the Fluorescent Enhancement of Ethidium Bromide Bound to DNA (see 6.4.2 for experimental conditions)

Table 7 Quenching of Ethidium Bromide-DNA Fluorescence by the Peptide Conjugates

Peptide Conjugate*	% Maximum Fluorescence Quenching**
4A	5.3
4B	11.1
4C	9.6
4G	25.1
7A	44.7
7B	14.0
7C	23.9
7G	8.5

*Refer to table 5 for peptide conjugate structures; **Determined from the addition of ethidium bromide aliquots to solutions containing i) DNA only and ii) DNA and peptide conjugate – the % of fluorescence quenched was determined from the maximum fluorescence of ethidium bound to DNA in the absence of drug and of ethidium bound to DNA in the presence of drug; See 6.4.2 for experimental conditions.

4.1.3 Thermal Denaturation Studies

Thermal denaturation, that is, unwinding of DNA in the presence of a drug leads to changes in the absorbance characteristics, as discussed earlier in 2.2.4. which can be monitored spectroscopically [58,59]. This phenomenon is used as an indicator of a drug/DNA affinity [58,59]. Figure 41 shows the melting curve of DNA in the presence of peptide conjugate 7A. The effect of the peptide conjugates on the thermal denaturation of calf thymus DNA at a drug to DNA ratio of 1:10 are presented in table 8.

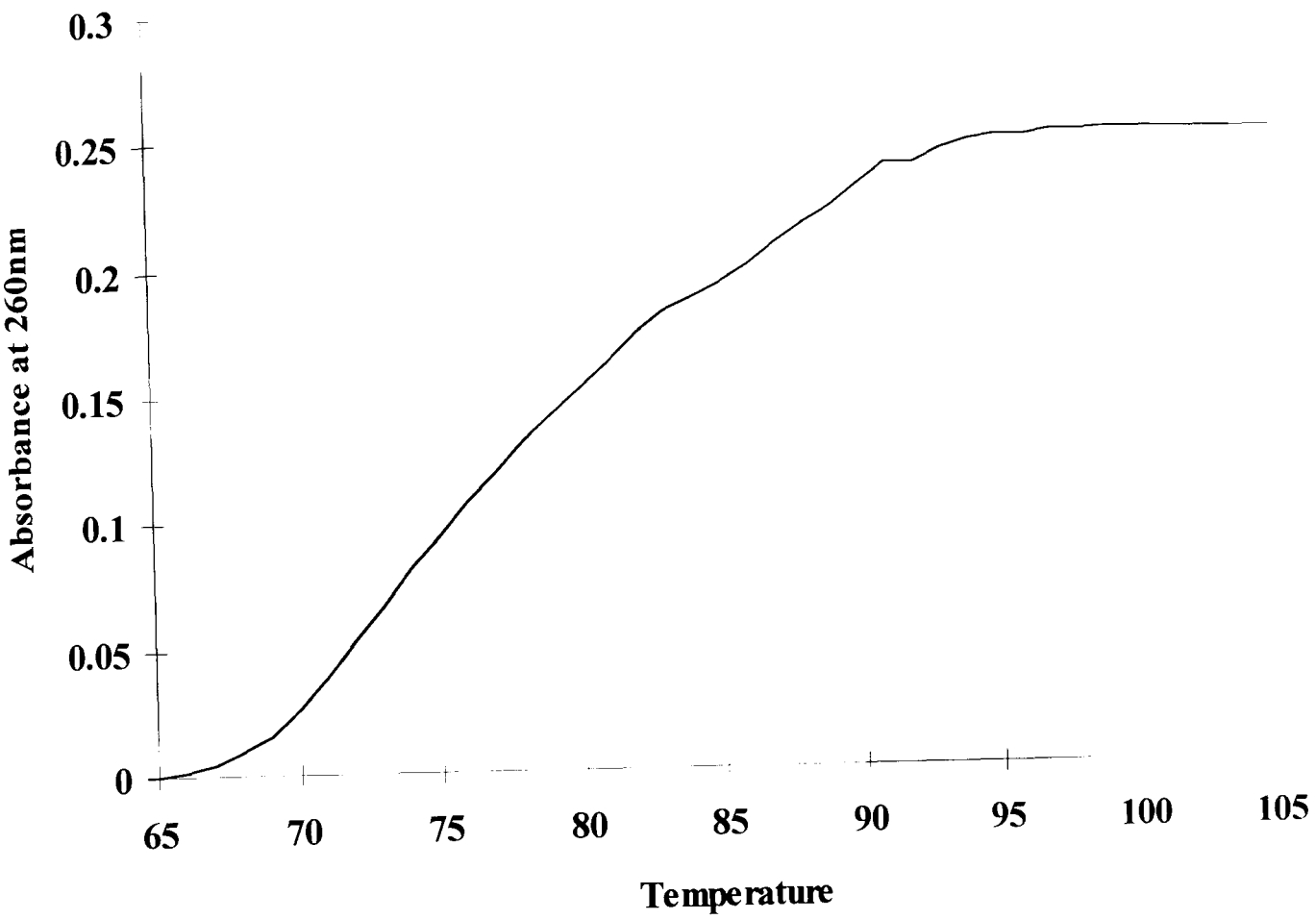


Figure 41 Thermal Denaturation of DNA in the Presence of Peptide Conjugate 7A (see 6.4.3 for experimental conditions)

Table 8 The Effect of Peptide Conjugates on the Melting Temperature of Calf Thymus DNA

	**ΔT_m °C			
*Peptide	*Intercalator-Linker			
	A	B	C	G
1	4.8	–	–	–
2	7.5	–	–	–
3	5.5	–	–	–
4	5.5	5.6	4.8	3.9
5	7.3	–	6.3	–
6	10.3	–	5.9	–
7	9.0	5.0	8.5	7.8
8	6.0	–	8.5	–

*Refer to table 5 for peptide sequences and peptide conjugate structure; **ΔT_m is the difference between the melting temperature of calf thymus DNA in the presence and absence of peptide conjugate at a 10:1 DNA/drug ratio; The melting temperature of calf thymus DNA was found to be 71.0°C (mean of duplicate results); The ΔT_m values presented in this table are the mean of duplicate results (± 0.5°C); – Not determined; See 6.4.3 for experimental conditions.

These results show stabilisation of the DNA double helix to heat denaturation in the presence of the peptide conjugates. It is evident that conjugates 6A, 7A (figure 32) and 7C are the most effective where as 4A, 4C and 4G are the least effective.

The ΔT_m values of some of the peptide conjugates tested are comparable to those of mono-substituted anthraquinones reported in the literature [57,76], (table 6, section 4.1.1). However compared to bis-substituted anthraquinones, the peptide conjugates are relatively weak intercalators indicating a low affinity of binding to DNA. It is important to note, however, that these studies primarily account for the binding of the intercalating chromophore of the conjugates and do not necessarily give any indication of the affinity of the peptide for DNA. These findings correlate with those obtained in the fluorescence studies which indicated that the peptide conjugates are relatively weak intercalators of DNA. It is possible that the peptides, most of which have been shown to bind DNA in a gel retardation assay (section 4.1.4), bind strongly to DNA without stabilising the double helix. It is interesting to speculate that the difference observed between activity in the electrophoretic mobility shift assay (section 4.1.4) which uses an AP-1 consensus sequence oligonucleotide and apparently for DNA binding using calf thymus DNA arise from the sequence selectivity of the peptides.

The results from both the thermal denaturation and fluorescence studies show that although the interaction of the peptide conjugates with calf thymus DNA is weak,

it is consistent with intercalation. These findings are consistent with those of Bailly *et al* who synthesised anilinoacridine derivatives containing the nucleic acid-binding tetrapeptide SPKK and demonstrated that these peptide hybrids intercalated into DNA [79]. The weak intercalative properties exhibited by the conjugates, synthesised in this study, is desirable as it ensures that the intercalating chromophore has the ability to move in and out of the double helix through reversible and non-specific interactions, thus aiding selectivity by allowing the peptide to effectively search the DNA for the AP-1 consensus sequence. It is anticipated that upon recognition of the AP-1 consensus sequence the drug will bind DNA with a greater affinity.

4.1.4 Electrophoretic Mobility Shift Assay [70]

Competition assays based on the electrophoretic mobility shift assay (EMSA) were conducted in an attempt to determine the selectivity and ability of the peptide conjugates to displace AP-1 from its DNA consensus sequence. The assays were conducted using cell nuclear fractions containing AP-1 family binding proteins and with recombinant c-jun homodimers. The EMSA was performed in the presence of dithiothreitol (DTT), a reducing agent. as the affinity of the fos and jun proteins for their cognate DNA sequences is dependent on the reduced state of the specific Cys residue of the highly conserved motif KCR. found in the DNA binding domains of these proteins [29]. Reduction of this Cys residue generates a free sulphydryl group which permits DNA binding of the AP-1 family of proteins and hence activates transcription and cell growth [11]. Since

the KCR motif was incorporated in the peptides employed DTT was also added to ensure that the Cys residue of this tri-amino acid sequence was maintained in a reduced state to allow DNA binding.

The extent of inhibition of the AP-1 protein by the peptides and their conjugates was determined by the intensity of the bands observed in the EMSA using a densitometer. The results have been summarised in table 9. A high band intensity indicated that the peptides/conjugates had very little or no effect on the displacement of the AP-1 protein, whereas a low band intensity indicated that the peptide/conjugate was an effective inhibitor.

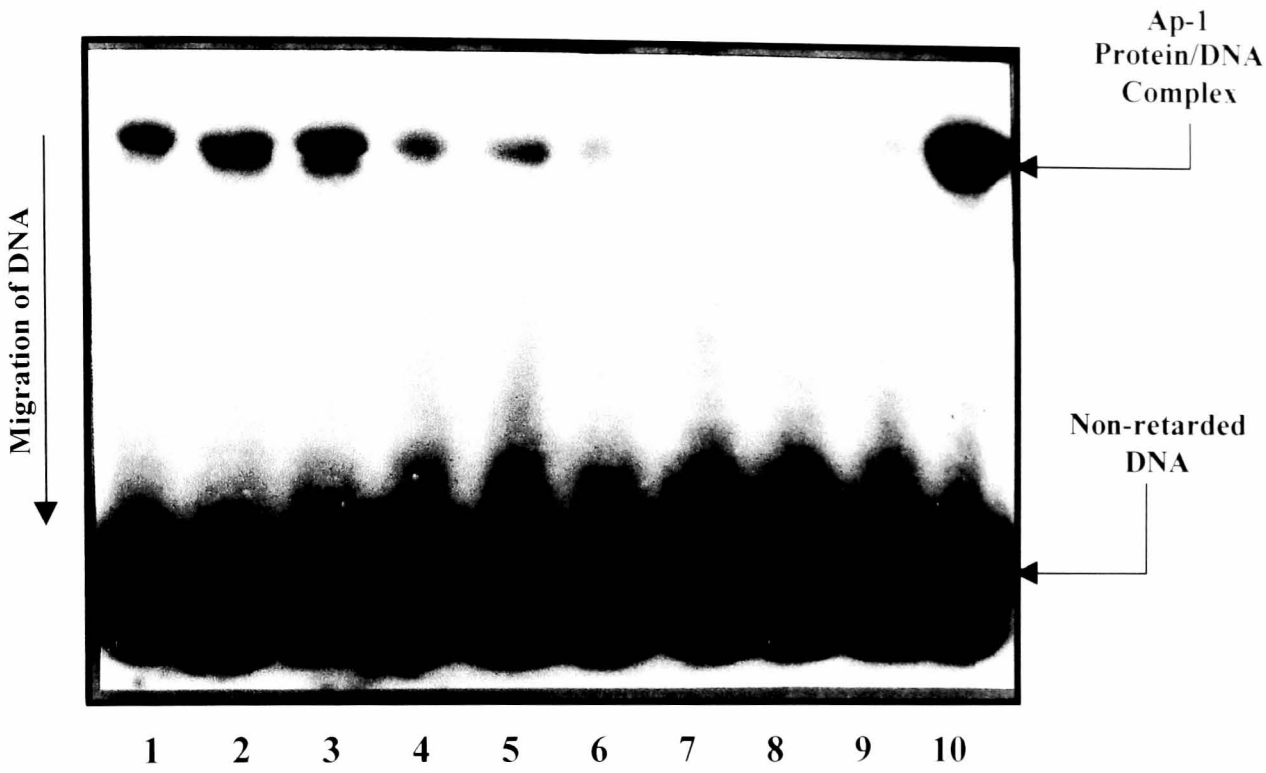
From the results obtained it was apparent that the peptides were less effective in preventing the DNA binding of the c-jun homodimer compared to the peptide conjugates, thus suggesting that the intercalating moiety enhances the effectiveness of the peptide and hence inhibition of the AP-1 transcription factor. The results also show that the peptide conjugates containing the more basic sequences 4, 5, 6 and 7 were the most effective in displacing the AP-1 protein from its DNA consensus sequence. In particular peptides 4 and 7 (AKCRNA and AKCRKRA respectively) and their respective conjugates exhibited the greatest biological activity. Figures 42 shows a typical EMSA of peptide conjugate 7A in which the DNA binding activity of this drug is clearly enhanced with increasing drug concentration. Although DTT was added to ensure the Cys residue was maintained in a reduced state to permit DNA binding of the peptides it is possible

that complete reduction of these moieties was not being achieved. This could explain why complete inhibition of the AP-1 protein was not observed with any of the peptides or conjugates and also why such high drug concentrations were necessary.

Table 9 The Effect of the Peptides and their Conjugates on the DNA Binding of the AP-1 Protein* [70]

	% Mean Band Intensity**			
	Peptide	Peptide Conjugate		
Peptide		A	C	D
1	98	51	-	-
2	99	43	-	-
3	99	41	-	-
4	82	32	-	-
5	83	39	35	42
6	87	38	34	39
7	56	26	23	-
8	79	56	53	-

* Displacement of the AP-1 protein from the DNA AP-1 consensus sequence was determined using EMSA [72]; ** Determined using a densitometer; The results are the mean value of two separate EMSAs for each compound. The concentrations used were: AP-1 = 10.73ng; DNA = 0.108ng = 2.1nM; Peptide = 16.8μM; Peptide conjugate = 16.8μM; - Not determined.



Lanes 1 & 10 Contain ^{32}P-labelled DNA AP-1 consensus sequence and the AP-1 (fos/jun heterodimer) protein (HeLa cell nuclear extract) with no added peptide conjugate 7A

Lanes 2-9 Contain ^{32}P-labelled DNA AP-1 consensus sequence (2.1nM) with an increasing concentration of peptide conjugate 7A (2: 0.21μM; 3: 0.42μM; 4: 0.63μM; 5: 0.84μM; 6: 1.05μM; 7: 1.26μM; 8: 1.47μM; 9: 1.68μM)

Figure 42 An Electrophoretic Mobility Shift Assay Showing the Effect of Peptide Conjugate 7A on the Displacement of the AP-1 Protein form the DNA AP-1 Consensus Sequence

The binding activity of peptides 4 and 7 and their conjugates observed in this study correlated with the results obtained from the circular dichroism (CD) and molecular modelling studies, which are discussed in greater detail in section 4.2. The CD studies suggested that these peptides (i.e. 4 and 7) possess an intrinsic propensity to form helical structures, a requirement that seems to be essential in allowing tight binding interactions between the AP-1 family of proteins and their cognate DNA sequences [21,80-82] (see section 4.2.2). Similarly, molecular modelling (see section 4.2.3) indicated that peptides 4 and 7, in particular, were more likely to form helical structures and hence bind the DNA AP-1 site with a greater affinity compared to peptides 1-3, 5, 6 and 8. Interestingly, displacement of the AP-1 protein by peptides 4 (AKCRNA) and 5 (AKCRKA) is similar, although it was expected that the substitution of Asn (N) in peptide 4 with Lys (K) in peptide 5 would enhance the non-sequence specific electrostatic interactions/binding with DNA. Although this seems to be apparent in the DNA thermal denaturation studies (see section 4.1.3), the gel retardation assay indicates that peptide 4 shows greater DNA AP-1 inhibition and hence demonstrates sequence selectivity.

4.2 Structural Evaluation

4.2.1 NMR Spectroscopy

4.2.1.1 COSY Experiments

COSY experiments were performed on all the peptides and their respective conjugates in DMSO. Complete structural elucidation using this technique, unfortunately was not possible from the spectra obtained. This was due to the presence of broad and unresolved peaks thought to be a result of poor environmental stability. In addition to this, strong overlapping of resonances, in particular those of the β- and γ- protons of the Arg and Lys residues meant that not all of the amino acid protons could be unambiguously assigned. Figure 43 shows a COSY spectrum of peptide 4 and table 10 shows the protons that were identified.

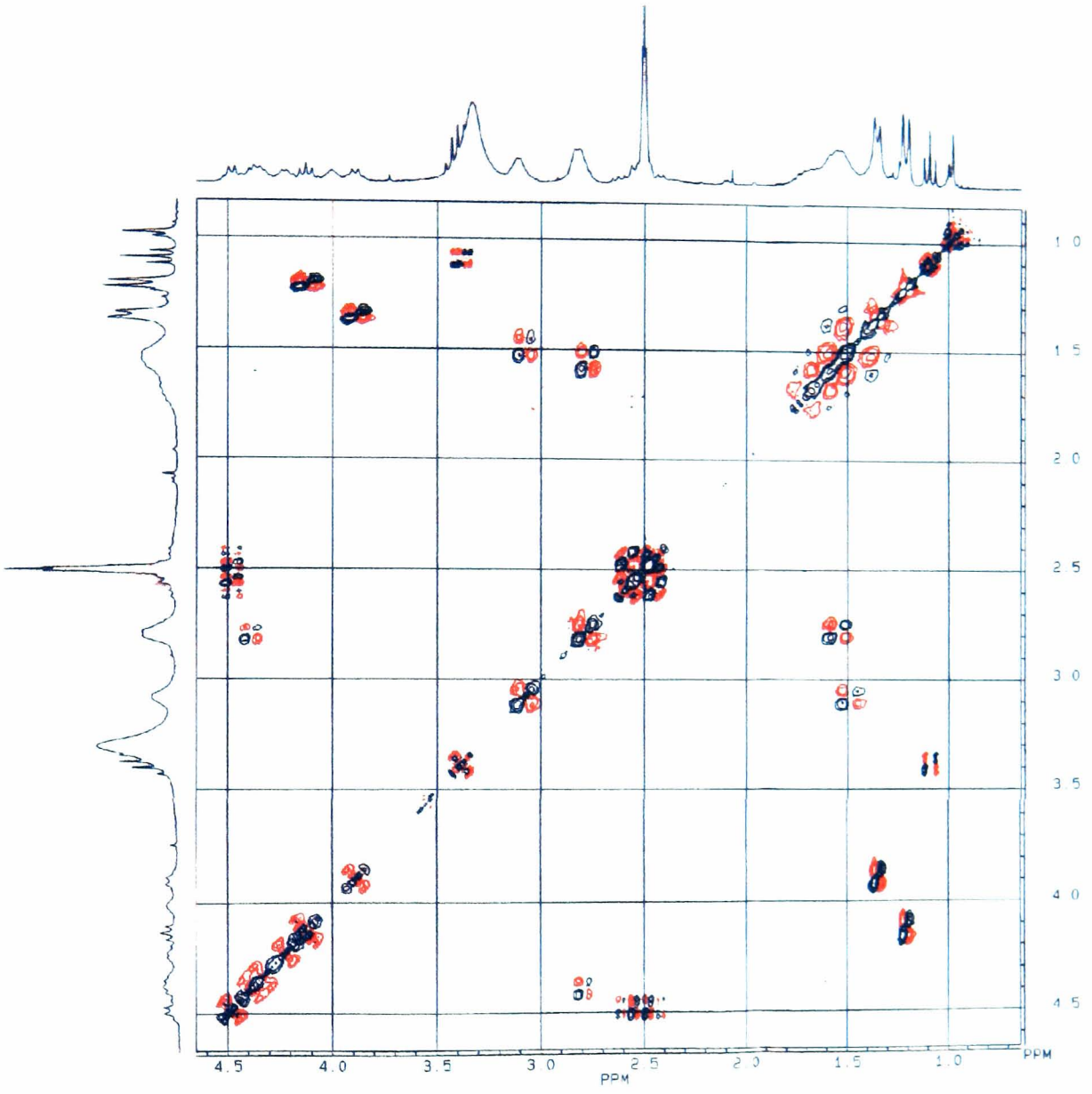


Figure 43 Partial ^{1}H-COSY Spectrum (0-4.5ppm) of Peptide 4 in DMSO (see 6.5.1 for experimental conditions)

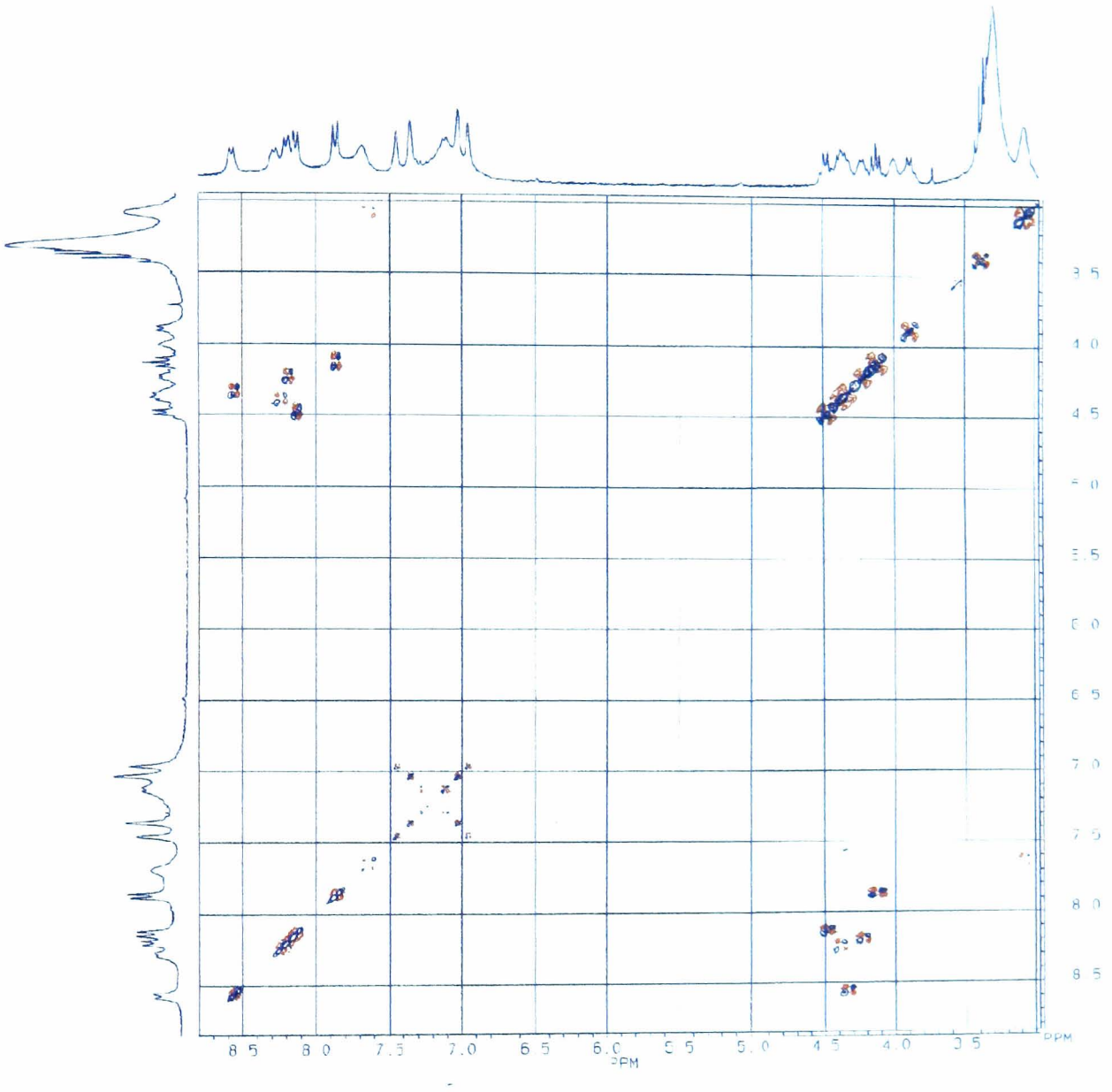


Figure 43 Continued Partial ^{1}H-COSY Spectrum (3.0-8.5ppm) of Peptide 4 in DMSO (see 6.5.1 for experimental conditions)

Table 10 ^{1}H-COSY Assignment of Peptide 4*

	Resonance (ppm)						
	NH	α	β	γ	δ	ε	Other
Ala	8.1	3.9	1.1				
Lys	8.2	4.2	1.6	(1.4)	1.6	2.8	
Cys	8.1	4.3	2.8				
Arg	(8.2)	(4.2)	(1.7)	1.4	3.1		7.0-7.3**
Asn	8.1	4.4	2.6				
Ala	7.9	4.15	1.3				

*COSY experiments conducted in DMSO (see 6.5.1 for experimental conditions); Figures in brackets are not fully resolved; **Guanidinium protons.

4.2.1.2 NOESY Experiments

NOESY experiments were performed on peptides 4 and 7 were analysed in DMSO and trifluoroethanol (TFE). The latter is a helix-inducing solvent, which stabilises helices in regions with some α-helical propensity [46,83]. It was anticipated that the data obtained from these experiments would establish whether these peptides were helical or at least whether they possessed an intrinsic propensity to form a helical structure. The spectra generated from the experiments conducted in both DMSO and TFE showed no conclusive evidence to suggest that the peptides were α-helical, although a correlation signal was

observed between the Lys ε-protons and the Arg guanidinium group which suggested some helicity.

These results were not entirely unexpected, as it had previously been shown in the CD studies, discussed in section 4.2.2, that the peptides only assumed helical structures upon transfer to a more hydrophobic environment. Unfortunately the spectra of the peptides in TFE also showed no coupling signals that were indicative of α-helicity. The lack of order i.e. secondary structure observed in these peptides however does not necessarily mean that the peptides are not helical. It is reasonable to say that the peptides are helical in TFE as shown in the CD studies (section 4.2.2) but the degree of helicity is relatively low, which is why it is not apparent from the NOESY experiments. Furthermore, many of the protons that might be expected to give cross-peaks (including the Cys SH, Lys NH_2 and Arg guanidinium group) undergo exchange with the deuterated TFE.

4.2.2 Circular Dichroism Studies [84]

Circular dichroism of peptides 4 and 7 and their corresponding conjugates were conducted by a research colleague [84]. It was anticipated that the peptides employed in this study would either adopt α-helical conformations or appear as random coils. A high degree of helicity was not expected since one α-helical turn incorporates approximately 3.6 residues [44] and the peptides under investigation were only of 5-7 residues in length. CD of the peptides and their conjugates was measured in 80% TFE/water and 100% TFE at 20°C. The spectra recorded in the

more aqueous environment were indicative of a random coil structure rather than an α-helical conformation. This was suggested by the band at 195nm which is characteristic of random coiling and also by the absence of the two distinct bands normally observed at 208 and 222nm which signify α-helicity [66]. The latter two bands, however were observed in the spectra of the peptides and their respective conjugates in 100% TFE (figure 44), thus suggesting that these peptides possess an intrinsic propensity to form helical conformations in hydrophobic environments.

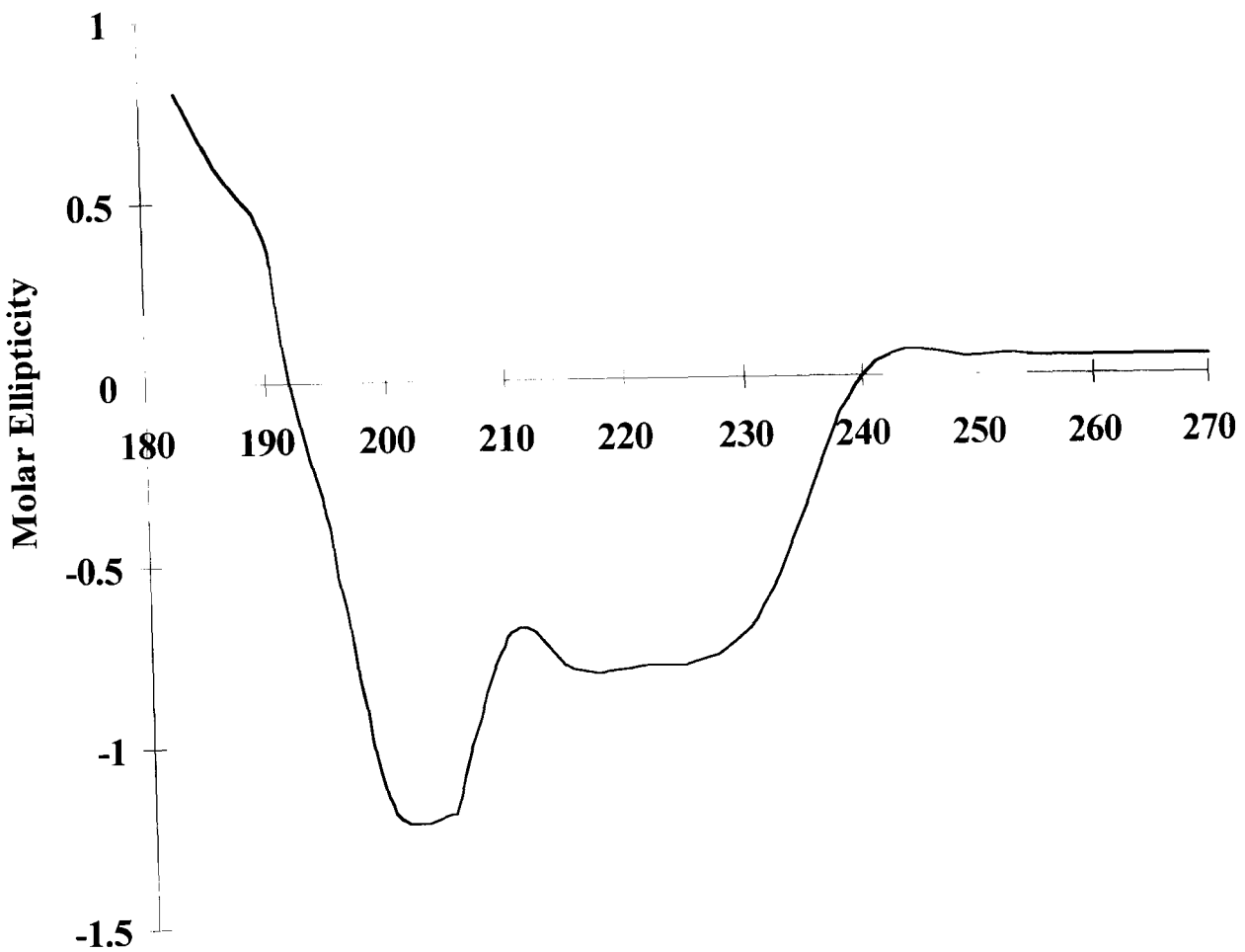


Figure 44 CD Spectrum of Peptide 4 (AKCRNA) in 100% TFE (see 6.5.2 for experimental conditions)

On the basis of the results obtained it can be concluded that both peptides 4 and 7 and their corresponding conjugates are predominantly random coil in aqueous solution, although some helical structure is observed upon transfer to a more hydrophobic environment. The lack of helical structure observed in an aqueous environment in these experiments does not preclude these peptides from adopting an α-helical conformation upon binding DNA. These results correlate with those obtained from studies of the basic regions of the bZip proteins for example GCN4, which in the absence of DNA exist predominantly in a random coil conformation with some helical tendencies. However upon binding to DNA, a more hydrophobic environment, these regions have been shown to assume α-helical structures [20,80-82]. The lack of helicity observed from the CD and NMR studies is due to size and also quite possibly to the absence of the DNA AP-1 consensus sequence which may be an important factor in inducing α-helicity [20,80-82].

4.2.3 Molecular Modelling

Insight II, a software program from Biosym Technologies was employed to conduct theoretical studies on the peptides and their interactions with the DNA AP-1 consensus sequence using the amber and cvff forcefields. Energy minimisations were performed on the peptides in an aqueous environment, both in their extended and helical conformations and also when complexed with the DNA AP-1 consensus sequence. It was anticipated that the energies obtained from these calculations would allow (i) the energy change for transformation of the

the peptide from its extended form to its α-helical binding conformation and (ii) the energy change for the interaction of the helical peptide with DNA. In other words, the energies obtained from these studies can be used to establish the likely conformation of the peptides and also determine whether the interaction between these peptides and the DNA AP-1 consensus sequence is favourable. The results acquired from these calculations are summarised in table 11.

Table 11 Calculated Energies of AP-1 Consensus Sequence Peptides in an Aqueous Environment (kcal mol^{-1})

*Energy Calculations	***Conformer	Peptide							
		1 ARCKA	2 AKCRA	3 AKSRA	4 AKCRNA	5 AKCRKA	6 AKCRNRA	7 AKCRKRA	8 AAKCRAA
Peptide Potential Energy	Extended	18.01	44.36	38.34	58.32	61.76	52.15	-22.70	44.51
	Bound (H)	124.94	40.83	36.28	55.71	51.22	54.64	-22.89	60.31
Peptide Solvation Energy	Extended	-202.91	-233.23	-231.51	-242.89	-248.89	-245.54	-269.57	-248.14
	Bound (H)	-219.96	-189.66	-191.58	-203.77	-207.01	-214.88	-248.82	-252.34
Extended to Helix Transformation**		89.88	40.04	37.87	35.73	31.34	33.16	20.56	11.55
Interaction Energy with AP-1 Oligonuleotide	Bound (H)	-576.63	-609.23	-607.83	-615.35	-611.95	-594.08	-612.23	-577.39
Relative Solvation Energy in Complex	AP-1 DNA	-2.4	29.95	40.95	30.19	43.24	3.61	45.19	5.34
	Bound (H)	104.38	98.58	89.37	90.04	88.22	104.04	67.77	100.79
Binding Energy		-474.65	-480.7	-477.51	-495.12	-480.49	-486.43	-499.27	-471.26

Calculated using an RMS derivative of 0.001kcal/mol and 5000 iterations; ** In solution; *** Peptide conformation; (H), Helical;

A = Ala C = Cys K = Lys N = Asn R = Arg S = Ser

It is apparent from the energies obtained that the heptamers, that is, peptides 7 and 8 are more likely to adopt α-helical structures, although in each case this process does not seem to be spontaneous. Peptide six which is also a heptamer however seems to require greater energy than peptides 7 and 8 to form a helical structure. This is thought to be due to the presence of an Asn residue which is known to disrupt α-helices because its side chain contains both a hydrogen bond donor and acceptor in close proximity to the peptide backbone, where they compete for the main chain NH and CO groups [85]. In general the binding energies of the peptides are similar and suggest that the interaction of these peptides with the DNA AP-1 site is spontaneous and hence favourable. Figure 45 shows the interaction of peptide 7 with the DNA AP-1 consensus sequence. From the results shown in table it can be deduced that peptides 4 and 7 are the two most strongly binding peptides. This correlates with the experimental findings using the EMSA (section 4.1.4). The next most affinic AP-1 peptide is 6, which has a binding energy 8.7kcal mol^{-1} (36.5 kJ mol^{-1}) lower than that of 4. Peptides 4 and 7 also show the strongest interaction energy with the DNA AP-1 consensus sequence (table 11). Aside from this, no clear pattern emerges from the molecular modelling data.

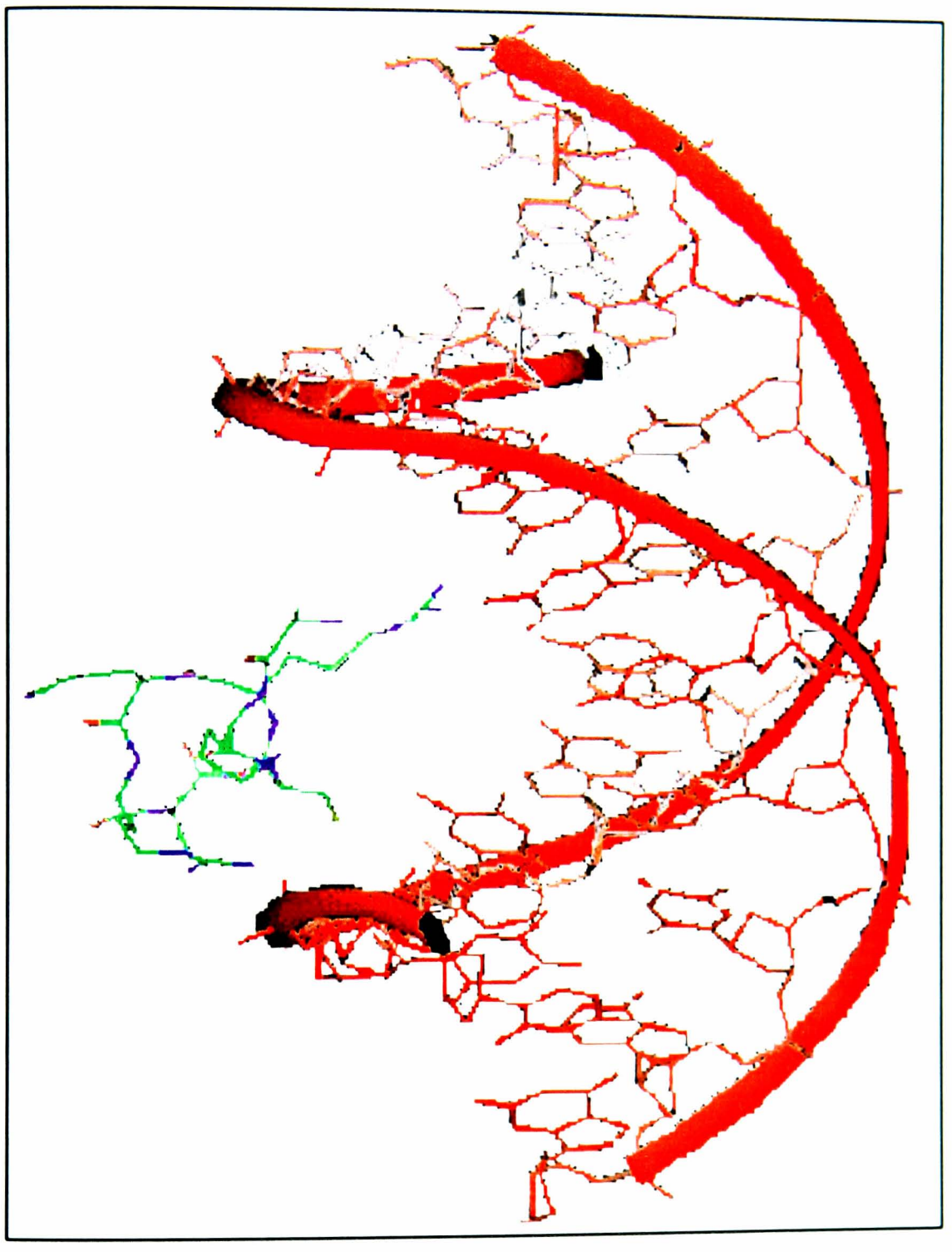


Figure 45 A Computer model of Peptide 7 Complexed with the DNA AP-1 Consensus Sequence (see 6.5.3 for experimental details)

Energy minimisations were also performed on peptide 9 in its constrained form when complexed with the AP-1 site (figure 46). The binding energy obtained from this calculation was -422.39kcal mol^{-1}. From this energy value, it is apparent that the interaction between this peptide and DNA is not as spontaneous as that between the AP-1 site and the unrestrained peptides, the binding energies of which ranged between -474 to –500kcal mol^{-1}. The difference in the binding energies of the unrestrained peptides and the constrained helical peptide was attributed to the presence of the alkanediamine bridge, which seemed to be hindering the contact of some of the residues of peptide 9 with the DNA AP-1 consensus sequence.

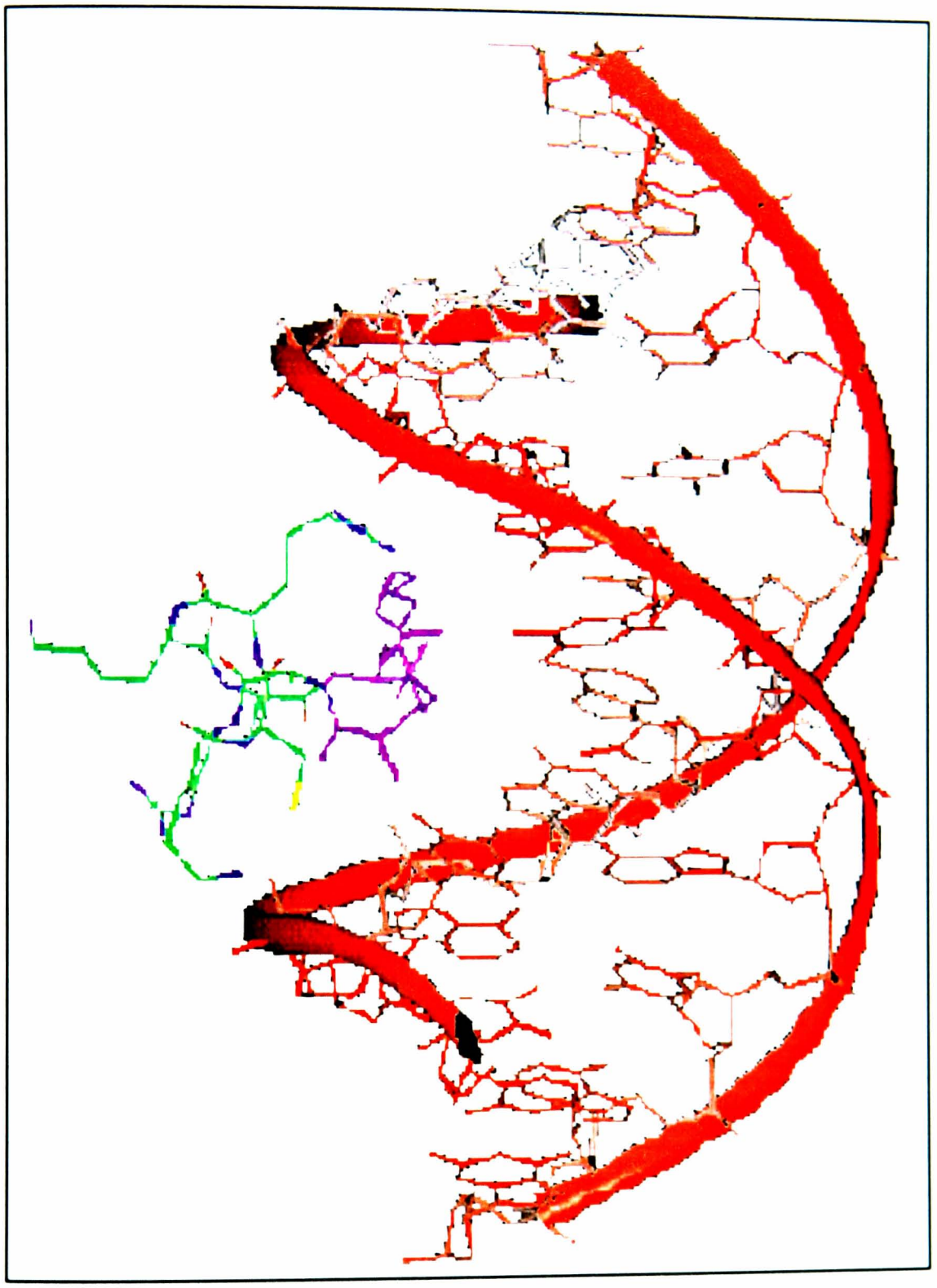


Figure 46 A Computer Model of Peptide 9 (see 3.7, figure 34, for peptide sequence) Complexed with the DNA AP-1 Consensus Sequence (See 6.5.3 for experimental details); The butyl constraint has been shown in purple

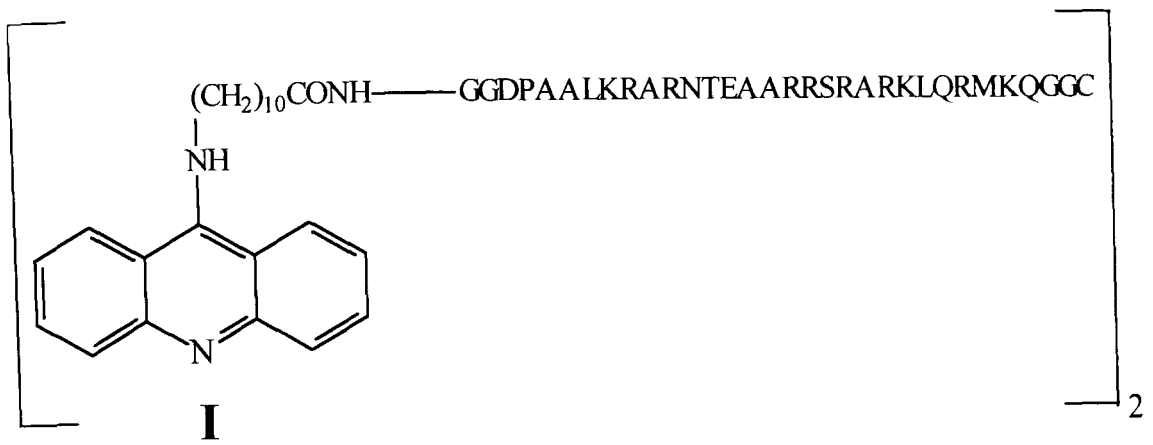
4.3 Summary

It can be concluded that the results obtained from the structural analysis of the peptides and the conjugates correlate with those from the biological studies. Experimental studies show that conjugates of peptides 4 and 7 are particularly effective in preventing the DNA binding of the c-jun homodimer (see section 4.1.4). The greater activity exhibited by these peptides may be attributed to the ability of the peptide moieties to form helical structures, which seem to be an important factor in the DNA binding of the AP-1 family of proteins (see section 4.2.2).

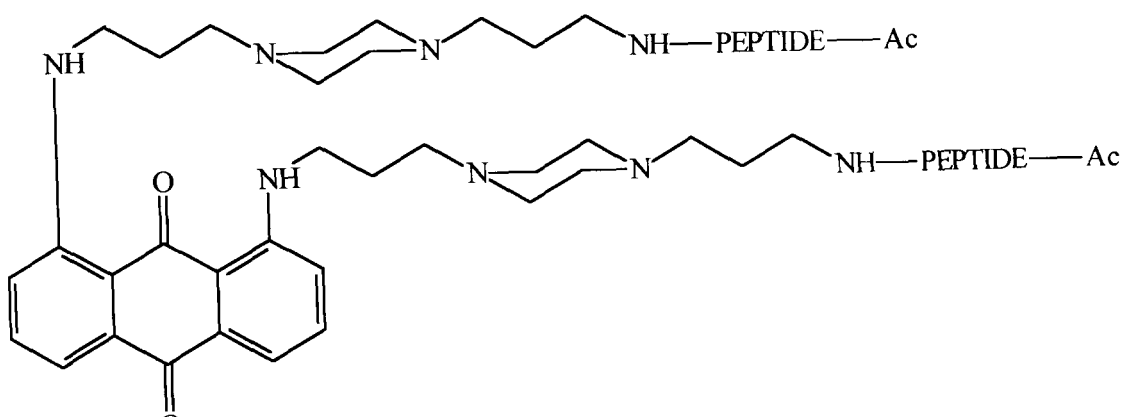
5 SUMMARY AND CONCLUSION

The use of synthetic peptides with an ability to recognise and bind to specific sequences of DNA has long been considered a useful probe to study the mechanism of gene expression and to potentially control transcription [79,86,87]. Based on this concept several intercalator-linked peptides, bearing the KCR motif found in the DNA binding domains of the AP-1 transcription factor were successfully synthesised. Biological analysis of these peptide conjugates demonstrated that short peptide fragments of 6-7 residues were capable of preventing the DNA binding of the AP-1 protein and hence potentially controlling transcription. Analysis of these conjugates using spectroscopic techniques established that they were weak intercalators of DNA and hence suggested that the DNA binding affinity of these potential transcription factors was governed principally by the peptide moiety. It is anticipated that the greater DNA binding of the peptide over the intercalating moiety would aid selectivity.

It is likely that both the inhibition of the AP-1 protein and selectivity of the peptide conjugates may be enhanced by incorporating the entire sequence found in the DNA binding domains of the fos and jun proteins including the second DNA binding chain found in the native fos/jun dimers. Since this work began, this approach has been described by Tabor [88] and Takenaka *et al* [89]. Both workers described the synthesis of GCN4 (the yeast equivalent of the AP-1) intercalator peptide hybrids which are shown in figure 47.



I



II

PEPTIDE = DPAALKRARNTEAARRSRARKLQRM

Figure 47 GCN4 Intercalator Hybrid Peptides I [88], II [89]

Compound (I), synthesised by Tabor [88] consists of the N-terminal basic region of the GCN4 transcription factor with a Gly-Gly-Cys motif added at the C-terminus to enable dimerisation via disulphide bond formation. This peptide was then attached to 9-chloroacridine via an 11-aminoundecanoic linker and two further glycine (Gly) residues. The peptide moieties in compound (II) also mimic

the basic region of GCN4. In this case however the peptides were connected to 1,8-bis[[4-(3-aminopropyl)piperazinyl]propylamino]-9,10-anthraquinone to give a dimeric model peptide. Takenaka *et al* [89] observed both a sequence specific interaction and increased α-helicity in the peptide moieties of (II) upon binding DNA using Circular Dichroism studies. Interestingly the biological evaluation of these peptide hybrids has not yet been reported. This may be because although the approach undertaken by Tabor [88] and Takenaka [89] would be useful in principle, it is unlikely to have practical application. Incorporation of the entire sequence found in the DNA binding domains of the fos and jun proteins, including the second chain, would possibly maximise the selectivity of an AP-1 inhibitor, but in doing so it would most likely create a number of difficulties. In the first instance, the task of synthesising such a large molecule and its management is likely to be difficult both in terms of purification and solubility. Secondly, the biological stability of such a large compound would be problematical, in particular because, the peptide chains would be readily susceptible to enzyme hydrolysis. Thirdly, cellular and nuclear localisation of these bulky molecules would be unlikely.

Unlike the work reported by Tabor [88] and Takenaka [89], the studies conducted in this project have demonstrated that peptide conjugates with relatively short linkers and peptide fragments containing the critical KCR motif are capable of inhibiting DNA binding of the AP-1 protein and also that incorporation of the entire sequence found in the DNA binding domains of the AP-1 transcription

factor is not necessarily required. In addition to inhibiting the binding of the AP-1 protein, the peptide conjugates are designed to be subject to redox regulation. This, in principle, ensures that inhibition of the AP-1 protein occurs only in cells under oxidative stress and anticipates therefore that the drug has very little or no effect on the transcription of normal healthy cells. The selectivity of these AP-1 inhibitors for tumours in oxidative stress where cellular oxygen and or reducing thiols are depleted however remains to be investigated.

5.1 Future Work

Further studies are required for a more extensive biological and structural evaluation of the peptides and their conjugates. Future works would include i) determining the DNA binding of the conjugates under oxidative conditions, i.e. determine the selectivity of the peptide conjugates for tumour cells under oxidative stress; ii) establish the binding affinity of the peptide conjugates for the DNA AP-1 consensus sequence and finally iii) measure the helical content of the peptides upon binding the DNA AP-1 consensus sequence using circular dichroism and NMR spectroscopy.

6 EXPERIMENTAL

The ^{1}H- and ^{13}C-NMR spectra were recorded on a 250MHz super-conducting Bruker AC250 Spectrometer at 303.3K unless otherwise stated. Infrared spectra were recorded in nujol muls or potassium bromide on a Perkin-Elmer 683 Spectrophotometer. The mass spectra were recorded on a VG 70 SEQ spectrometer and MALDI-MS was performed using a Finnigan MAT Laser Desorption spectrometer. The elemental analysis was conducted at the Microanalytical laboratory at Manchester university.

Melting points were determined on a hot stage microscope and are uncorrected. Thin layer chromatography was performed on silica gel plates and was monitored sequentially with UV light and iodine powder; ninhydrin spray and bromocresol green were employed to stain amino and carboxyl groups respectively. Silica gel 60 (220-440 mesh) was employed where column chromatography was used.

6.1 Synthesis of Intercalator-Linkers

Synthesis of 1-[*N*-{2-Aminoethyl}amino]anthraquinone

1-Chloroanthraquinone (250mg; 1.0mmol) was dissolved in excess ethylenediamine (5g; 0.083mol) and was left to stir at room temperature under nitrogen for 72hrs. The reaction mixture was diluted with water (100ml) to yield a red precipitate. This was purified using column chromatography, eluting with

dichloromethane and an increasing concentration of methanol (0-50%), giving the product as a red solid (100mg; 36.5%); Mpt 148°C: ν_{max}/cm^{-1} 3350 (NH_2), 2590 (CH), 1690 (C=O), 1600 (C=C), 1280 (NH), 700 (ArCH); δ_H (CD_3OD): 3.0 (m, 2H; C*H*$_2$$NH_2$), 3.5 (t, 2H; NHC*H*$_2$), 7.25 (d, 1H; Ar*H*), 7.5 (m, 2H; Ar*H*), 7.8 (m, 2H; Ar*H*), 8.15 (d, 2H; Ar*H*), 8.3 (d, 1H; Ar*H*); δ_C (CD_3OD): 41.86, 43.25, 116.67, 119.31, 127.0, 127.77, 134.29, 135.01, 136.55, 144.5, 181.5, 183.27; m/z $(M + H)^+$ 267; CHN: Found C (68.99%) H (5.33%) N (9.82%), Calculated with 0.75 H_2O, C (68.69%) H (5.55%) N (10.02%)

Synthesis of 1-[*N*-{2-Succinamidylethyl}amino]anthraquinone

1-(*N*-{2-Aminoethyl}amino)anthraquinone (100mg; 0.37mmol) and succinic anhydride (37.5mg; 0.45mmol) were dissolved in DMF (10ml) and left to stir for 3hrs under nitrogen at room temperature. The reaction mixture was diluted with water (50ml) and the product was extracted with dichloromethane (3 x 25ml). The organic layers were dried over $MgSO_4$ and reduced *in vacuo*. The product was chromatographed, eluting with dichloromethane and an increasing gradient of methanol (0-50%) and was isolated as a red solid (137.5mg; 72%); Mpt 156°C: ν_{max}/cm^{-1} 3400 (COOH), 3300 (NH), 2900 (CH), 1710 (C=O), 1680 (NHC=O), 1630 (C=C), 1280 (NH), 700 (ArCH); δ_H (DMSO): 2.4 (m, 4H; NHC*H*$_2$C*H*$_2$$NH_2$), 3.2 (t, 2H; COC*H*$_2$), 3.4 (t, 2H; C*H*$_2$COOH), 7.3 (d, 1H; Ar*H*), 7.4 (d, 1H; Ar*H*), 7.8 (m, 2H; Ar*H*), 8.1 (m, 2H; Ar*H*), 9.7 (t; 1H; Ar*H*); δ_C (DMSO): 40.25 113.0, 115.29, 126.38, 126.54, 132.46, 134.14, 134.5, 134.63, 135.71, 151.01, 151.67,

183.01, 184.0; m/z $(M + H)^+$ 367; CHN: Found C (64.93%) H (5.04%) N (7.56%), Calculated 0.75 H_2O, C (64.78%) H (4.99%) N (7.56%)

Synthesis of 1-[2-{*N*-(2-Aminoethyl]succinamidyl}ethyl)amino]anthraquinone

Ethylenediamine (16mg; 0.27mmol), PyBOP (142mg; 0.27mmol) and DIEA (47µl; 0.27mmol) were added to 1-[*N*-{2-lsuccinamidylethyl}amino]-anthraquinone (100mg; 0.27mmol) in DMF (15ml). This was then left to stir at room temperature, under nitrogen for 3hrs. The reaction mixture was reduced *in vacuo* and the product was chromatographed with an eluent of dichloromethane and an increasing gradient of methanol to yield a red solid (19mg; 17%); Mpt 171°C; ν_{max}/cm^{-1} 3300 (NH_2), 3050 (ArCH), 2980 (CH), 1680 (NHC=O, C=O), 1600 (C=C), 1290 (NH), 720 (ArCH); δ_H (CD_3OD): 2.2 (s, 4H; $COCH_2CH_2CO$), 3.0 (t, 4H; $ArNHCH_2CH_2$), 3.4 (m, 4H; $HNCH_2CH_2NH_2$), 7.3 (m, 2H; Ar*H*), 7.6 (m, 1H; Ar*H*), 7.9 (m, 2H; Ar*H*), 8.1 (m, 2H; Ar*H*), 9.7 (m, 1H; N*H*); δ_C (DMSO): 35-45, 115.17, 118.59, 126.25, 126.43, 133.44, 133.99, 134.37, 134.49, 135.56, 151.42, 171.49, 172.09, 182.88, 183.91; m/z $(M + H)^+$ 409; CHN Found C (61.56%) H (5.89%) N (12.79%), Calculated with 1.1 H_2O, C (61.71%) H (6.12%) N (13.09%)

Synthesis of 1-[*N*-{2-(2-Succinamylethyl)succinamidyl}ethylamino]anthra-quinone

Succinic anhydride (73mg; 0.73mol) was added to 1-[2-{*N*-(2-aminoethyl)succinamidyl}ethylamino]anthraquinone (250mg; 0.61mol) in DMF

(5ml) and left to stir for 18hrs under nitrogen at room temperature. The reaction mixture was diluted with water (25ml) to yield a red precipitate which was isolated by filtration. This was then chromatographed with an eluent of dichloromethane and an increasing gradient of methanol (30-100%) and was isolated as a red solid (80mg; 25.7%); Mpt 241°C; ν_{max}/cm^{-1} 3450-2400 (COOH), 3300 (NH), 3050 (ArCH), 2910 (CH), 1680 (NHC=O, C=O), 1570 (C=C), 1300 (NH), 700 (ArCH); δ_H (CD_3OD): 2.5 (t; $(CH_2)_2$NHCO; NH$(CH_2)_2NH_2$), 3.2 (t; 4H; CO$(CH_2)_2$CO), 3.5 (m; 4H; $(CH_2)_2$COOH), (7.3, d; Ar*H*), (7.5, m; ArH_2), (7.7, m; ArH_2), (8.2, d; Ar*H*), (8.3, d; Ar*H*); δ_C (DMSO): 35-40, 112.30, 115.19, 126.29, 126.50, 133.41, 133.49, 134.04, 134.44, 134.58, 135.65, 151.51, 171.53, 172.14, 172.49, 182.98, 183.95; m/z $(M + H)^+$ 509; CHN Found C (59.91%) H (5.21%) N (10.45%), Calculated with 0.7 H_2O, C (59.93%) H (5.64%) N (10.75%)

Synthesis of 2-[*N*-{2-Hydroxyethyl}-N-methylamino]ethylamine

Method A

2-Bromoethylamine hydrobromide (30g; 0.15mol) was added slowly to 2-(methylamino)ethanol (54.9g; 0.73mol) under nitrogen at room temperature, after which the reaction was refluxed for 5hrs. The reaction mixture was neutralised using 50% NaOH and the desired product was isolated by fractional distillation at 80°C at 1mmHg as a yellow oil (10.1g; 58%).

Method B

N-Methylenediamine (8.14g; 0.11mol) and boron trifluoride etherate (1.12g; 0.011mol) were added to benzophenone (20g; 0,11mol) in xylene (50ml). This was refluxed for 20hrs and the water produced during the reaction was removed using a Deane-Starke apparatus. The solvent was removed *in vacuo* to afford the desired product (24.6g; 0.103mol) which was used without further purification. Ethylene oxide (11.32g; 0.26mol) was then added to the benzophenone protected amine in methanol (50ml) at 0°C and refluxed using a condenser and a cold finger. The benzophenone protecting group was then removed by stirring the product with hydrochloric acid (100ml; 5M) for 18hrs at room temperature.

The reaction mixture was washed with dichloromethane (3 x 50ml) and then neutralised with sodium hydroxide. The product was finally extracted with dichloromethane (3 x 50ml) after which the organic layers were combined, dried over magnesium sulphate and then reduced *in vacuo*. The product was isolated as a yellow oil (7.8g; 60.2%) after purification by fractional distillation at 80°C at 1mmHg; ν_{max}/cm^{-1} 3300 (OH, NH_2), 2850 (CH), 1300 (NCH_3); δ_H ($CDCl_3$): 2.2 (s, 3H; N*CH*$_3$), 2.4 (m, 4H; *CH*$_2$N(CH_3)*CH*$_2$), 2.7 (t, 2H; NH_2*CH*$_2$), 3.5 (t, 2H; *CH*$_2$OH); δ_C ($CDCl_3$): 40.18, 42.13, 58.81, 59.12, 59.81; m/z $(M + H)^+$ 119

Synthesis of 1-[2-{*N*-(Hydroxyethyl)-N-methylamino}ethylamino]anthra-quinone

1-Chloroanthraquinone (2.9g; 0.012mol) and 2-[*N*-{2-Hydroxyethyl}-N-methylamino]ethylamine (14.6g; 0.12mol) were refluxed under nitrogen for 24hrs. The reaction mixture was diluted with water (150ml) and the product was extracted with ethyl acetate (3 x 100ml). The organic layers were combined, dried over $MgSO_4$ and reduced *in vacuo*. The product was chromatographed, eluting with dichloromethane and an increasing gradient of methanol (0-30%) to yield a red solid (340mg; 12.2%); Mpt 72°C; ν_{max}/cm^{-1} 3400 (OH), 3250 (NH), 2850 (CH), 1700 (C=O), 1620 (C=C), 1500(CH), 1190 (NCH_3), 1270 (NH), 700 (ArCH); δ_H ($CDCl_3$): 2.4 (s, 3H; N*CH*$_3$), 2.65 (t, 2H; NHCH$_2$*CH*$_2$NCH$_3$), 2.8 (t, 2H; N(CH$_3$)*CH*$_2$CH$_2$OH), 3.4 (q, 2H; NH*CH*$_2$CH$_2$N), 3.7 (t, 2H; CH$_2$*CH*$_2$OH), 7.0 (d, 2H; Ar*H*), 7.5 (m, 2H; Ar*H*), 7.65 (m, 2H; Ar*H*), 8.2 (t, 1H; Ar*H*), 8.25 (t, 1H; Ar*H*); δ_C ($CDCl_3$): 40.15, 40.83, 55.98, 59.07, 59.94, 115.70, 117.99, 126.65, 127.01, 133.04, 133.94, 134.81, 135.01, 135.43, 151.62, 183.88, 184.99; m/z (M + H)$^+$ 325; CHN: Found C (70.46%) H (6.26%) N (8.23%), Calculated with 0.025 H_2O, C (70.27%) H (6.18%) N (8.62%)

Synthesis of the 1-[2-{*N*-(Hydroxyethyl)-N-methylamino}ethylamino]anthra-quinone Hydrochloride

Ethereal hydrogen chloride (2ml) was added to 1-[2-{*N*-(hydroxyethyl)-N-methylamino}-ethylamino]anthraquinone (340mg;) in dichloromethane (5ml). The orange-red product was isolated by filtration and dried *in vacuo* for 18hrs.

Synthesis of 1-[2-{*N*-(2-Chloroethyl)-N-methylamino}ethylamino]anthra-quinone

Triphenylphosphine (157mg; 0.6mmol) was added to 1-[2-{*N*-(hydroxyethyl)-N-methyl-amino}ethylamino]anthraquinone hydrochloride (110mg; 0.3mmol) in dichloromethane (5ml). This was followed by the addition of carbon tetrachloride (276mg; 1.8mmol) after which the reaction was left to stir under nitrogen at room temperature for 20hrs. The product was isolated *in vacuo* and then purified using column chromatography eluting with dichloromethane and an increasing gradient of methanol (20%-80%), giving an orange-red solid (78.6mg; 68%); ν_{max}/cm^{-1} 3400 (NH), 2910 (CH), 1690 (C=O), 1590 (C=C), 1295 (NCH_3), 720 (Cl), 710 (ArCH); δ_H (MeOH) 3.1 (s, 3H; NCH_3) 3.55 (t(b), 2H; $NHCH_2CH_2NCH_3$) 3.7 (t(b), 2H; $N(CH_3)CH_2CH_2Cl$) 3.9 (t, 2H; $NHCH_2$) 4.0 (t, 2H; CH_2Cl) 7.3 (d, 1H; Ar*H*) 7.6 (m, 2H; Ar*H*) 7.5 (m, 2H; Ar*H*) 8.2 (dd, 1H; Ar*H*) 8.3 (dd, 1H; Ar*H*); δ_C (CD_3OD): 38.40, 38.52, 41.77, 56.02, 58.66, 115.39, 118.96, 127.60, 127.85, 134.66, 135.43, 136.0, 136.11, 136.89, 151.94, 184.66, 186.57; m/z $(M + H)^+$ 343

Synthesis of 9-[*S*-{Carboxymethyl}mercapto]anthraquinone

Thioglycolic acid (29mg; 0.56mmol) and DIEA (131mg; 0.56mmol) were added to 9-chloroacridine (100mg; 0.468mmol) in methanol (3ml). The reaction was left to stir at room temperature, under nitrogen for 18hrs. A precipitate was isolated by filtration and then chromatographed with an eluent of dichloromethane and an increasing gradient of ethanol (5-50%), giving a yellow powder (98mg; 77.7%); Mpt 211 °C; ν_{max}/cm^{-1} 3400-2400 (COOH), 2950 (CH), 2500 (SH), 1690 (CO),

1580 (C=C), δ_H (CD3OD): 3.7 (s, 2H; S*CH*$_2$), 7.5 (m, 4H; Ar*H*), 8.1 (d, 2H; Ar*H*), 8.9 (d, 2H; Ar*H*); δ_C (CD_3OD): 45.30, 127.99, 128.29, 129.85, 132.03, 149.53, 162.37; m/z (M^+) 298

Synthesis of BocLysine(Fmoc)OH

Fmoc-N-hydroxysuccinamide (342mg; 1.0mmol) in 1,4-dioxane (5ml) was added dropwise to BocLysine-OH in 10% Na_2CO_3 (5ml) at 0°C under nitrogen, after which the reaction was allowed to warm up to room temperature and then left to stir for 2.5hrs. The reaction mixture was diluted with water (20ml) and washed with ether (3 x 10ml) and dichloromethane (3 x 10ml). The aqueous layer was then acidified to pH2 using concentrated hydrochloric acid to yield a white solid (250mg; 52.6%); Mpt 62°C; ν_{max}/cm^{-1} 3500-2400 (COOH), 3350 (NH_2), 2950 (CH), 1700 (COOH, OCO), 1530 (C=C), 750 (ArCH); δ_H ($CDCl_3$): 1.6 (s(b), 13H; C*(CH*$_3$*)*$_3$; γ-*CH*$_2$; δ-*CH*$_2$), 1.8 (m(b), 2H; β-*CH*$_2$), 3.1 (m, 2H; ε-*CH*$_2$), 4.1 (d(b), 2H; α-*CH*; *CH*CH_2O), 4.4 (d(b), 2H; CH*CH*$_2$O), 7.2 (m, 4H; Ar*H*), 7.5 (d, 2H; Ar*H*), 7.7 (d, 2H; Ar*H*); δ_C ($CDCl_3$): 28.35, 47.29, 66.69, 67.09, 119.99, 125.07, 127.09, 127.15, 141.36, 143.99, 156.70; m/z $(M + H)^+$ 469

Synthesis of N-(4-Aminobutyl)phthalimide

Phthalic anhydride (252mg; 1.9mol) and 1,4-butadiamine (150mg; 1.9mol) were refluxed in chloroform (30ml) for 5hrs. The reaction mixture was allowed to cool and a white solid was isolated. This was purified using column chromatography with dichloromethane and an increasing gradient of methanol (5-50%). The

product was isolated as an off-white solid (75mg; 20.2%); Mpt 210-2°C; ν_{max} cm^{-1} 3400 (NH_2), 3100 (CH), 2900 (CH), 1650 (C=O), 1390 (N), 790 (ArCH); δ_H (D_2O): 1.7 (m, 4H; N$CH_2CH_2CH_2$), 3.0 (t, 2H; $CH_2CH_2NH_2$), 3.3 (q, 2H; NCH_2CH_2), 7.4 (m, 4H; Ar*H*); δ_C (D_2O): 26.98, 28.17, 41.81, 42.02, 129.94, 130.66, 132.16, 133.12, 182.36; m/z $(M + H)^+$ 219

Synthesis of 9-[2-{Hydroxyethyl}amino]acridine

9-Chloroacridine (100mg; 0.46mmol) was added to excess ethanolamine (10ml;) and left to stir at 80°C for 30hrs. The reaction mixture was diluted with water (50ml) to yield a yellow precipitate which was filtered and left to dry in a vacuum desiccator. The product was purified using column chromatography with an eluent of dichloromethane and an increasing concentration of ethanol (0-30%), and was isolated as a yellow powder (43mg; 38.7%); Mpt 196°C; ν_{max}/cm^{-1} 3495 (OH), 3310 (NH), 2800 (CH), 1650 (C=C), 750 (ArCH); δ_H (CD_3OD): 3.2 (t, 2H; NHCH_2), 4.0 (t, 2H; CH_2OH), 6.5 (m, 2H; Ar*H*), 6.8 (m, 1H; Ar*H*), 7.1 (d, 2H; Ar*H*), 7.5 (d, 2H; Ar*H*); δ_C (CD_3OD): 42.33, 45.50, 124.96, 125.71, 131.02, 132.99, 138.06, 167.44; m/z $(M + H)^+$ 239

6.1.1 Attempted Synthesis

Synthesis of 1-[*N*-{3-Carboxypropyl}amino]anthraquinone

1-Chloroanthraquinone (500mg; 2.06mmol), γ-aminobutyric acid (1.06g; 10mmol), zinc chloride (28.0mg; 0.2mmol) and 1M NaOH (10.3ml; 10mmol)

were refluxed in DMSO under nitrogen for 18hrs. The reaction mixture was cooled, diluted with water (100ml) and neutralised with concentrated hydrochloric acid to yield a red precipitate. Purification of this red solid was unsuccessful.

The above method was also employed in the synthesis of 1-(**S**-{carboxymethyl}mercapto)anthraquinone and 1-(*N*-methyl-N-carboxymethyl)-anthraquinone.

Synthesis of 9-[*N*-{Aminoethyl}amino]acridine

9-Chloroacridine (100mg; 0.46mmol) was dissolved in excess ethylenediamine (2.76g; 0.046mol) and was left to stir for 18hrs at room temperature. The reaction mixture was diluted with water (25ml) and the product was extracted with dichloromethane (3 x 20ml). The organic layers were combined, dried over magnesium sulphate and then dried *in vacuo*. Column chromatography was employed to purify the product using dichloromethane with an increasing gradient of methanol (10-50%). NMR analysis of the product however indicated that the reaction had been unsuccessful.

Synthesis of 9-[2-{Ethyl-*p*-toluenesulphonate}amino]acridine

p-Toluenesulfonyl chloride (142mg; 0.74mmol) was added to 9-[2-{hydroxyethyl}amino]acridine (100mg; 0.37mmol) in pyridine (2ml) at 0°C. The reaction mixture was gently swirled and then left to stand at 5°C for 24hrs. The mixture was diluted with ice-water and the isolated precipitate was washed with

water. The product was purified using column chromatography, however NMR analysis of the product suggested that the desired product had not been isolated.

6.2 Synthesis of the Peptides and their Conjugates

General Procedures for the Synthesis and Isolation of the Peptides and their Corresponding Conjugates

The peptides synthesised on the NovaSyn Gem synthesiser and those supplied by Calbiochem-Novabiochem Ltd were fully protected and bound to resin. The DMF was freshly distilled and TFA employed in all reactions was anhydrous and of HPLC grade respectively. All peptides and conjugates were stored at -20°C.

6.2.1 General Procedure For SPPS

The resin (1g) was solvated in DMF (10ml) for 45min prior to removal of the Fmoc protecting group using 20% piperidine in DMF, after which it was washed thoroughly with DMF. Activation of the Fmoc protected amino acid residue (4eq) was achieved using PyBOP (4eq) and DIEA (8eq) in DMF (5ml) – this cocktail was then added to the reaction vessel and left to react with free amino terminal of the resin for 1-2hr. The excess reagents were then removed by washing the resin with DMF and the residue was deprotected using 20% piperidine in DMF. This procedure was repeated until the desired peptide sequence had been completed.

6.2.2 General Procedure For Solution Phase Peptide Synthesis

The Fmoc-protected carboxyl component (1.00g; 2.1mmol), NMM (0.23ml: 2.1mmol), and EDC (400mg; 2.1mmol) were dissolved in anhydrous THF(50ml) under nitrogen and cooled to 0°C. The reaction was left to stir for 15minutes after which the amino component (290mg; 2.1mmol) was added. The reaction was left to stir at 0°C for a further 45minutes and then left to stand for 18hrs at 5°C.

The solvent was removed *in vacuo* and the resulting residue was dissolved in dichloromethane (100ml) and then washed with water (3 x 50ml), potassium hydrogen sulphate (3 x 50ml), sodium bicarbonate (3 x 50ml) and sodium chloride (3 x 50ml). The organic layers were dried over $MgSO_4$ and reduced *in vacuo*. The peptidic intermediates were purified either by column chromatography or recrystallisation.

Fmoc Deprotection

A 20% solution of piperidine in DMF was added to the resin at room temperature under nitrogen. This was left to stir for 1hr. The reaction mixture was then filtered and washed sequentially with DMF, acetic acid, dichloromethane and ether. The resin was dried in a vacuum desiccator and then stored at -20°C.

Final Deprotection and Cleavage

A solution of EDT (5%), TIS (5%) and water (5%) in TFA was added to the resin. This was left to stand for 5hrs with occasional swirling at room temperature.

under nitrogen. The resin was filtered and washed with TFA. The filtrate was reduced *in vacuo* at room temperature. The product was subsequently triturated with cold anhydrous ether and the dried in a vacuum desiccator over phophorous pentoxide (P_2O_5).

6.2.3 Conjugate Synthesis

The selective and final deprotection and cleavage of all conjugates was achieved using the conditions and methods described earlier. In each case prior to alkylation and amidation the resin was allowed to swell in DMF at room temperature under nitrogen for 45 minutes.

A - D and H Conjugates

The intercalator-linked acid (4eq), PyBOP (4eq) and DIEA (8eq) in DMF was added to the resin (1eq) at room temperature under nitrogen. The reaction mixture was left to stand for 3hrs with occasional swirling. The resin was washed with DMF, ethanol and ether. The resin bound conjugate was dried in a vacuum desiccator over P_2O_5.

E and F Conjugates

The mustard (4eq) and DIEA (8eq) in DMF was added to the resin (1eq) at room temperature under nitrogen. The reaction was left to stand for 3hrs with occasional swirling. The resin was washed with DMF, ethanol and ether and then dried over P_2O_5.

C Conjugates

BocLys(Fmoc)OH (4eq), PyBop (4eq) and DIEA (8eq) in DMF was added to the resin at room temperature under nitrogen. The reaction was allowed to stand for 3hrs with occasional swirling. The resin was washed with DMF, ethanol and ether. The Fmoc group was removed and 1-(N-{2-succinamidylethyl})-amino)anthraquinone (A) was then attached to the peptide using the method employed in the synthesis of the A conjugates.

D Conjugates

BocLys(Fmoc)OH (4eq), PyBop (4eq) and DIEA (8eq) in DMF was added to the resin at room temperature under nitrogen. The reaction was allowed to stand for 3hrs with occasional swirling. The resin was washed sequentially with DMF, ethanol and ether. The Fmoc group was then removed using the method described earlier.

The addition of BocLysOH was repeated after 1-(N-{2-succinamidylethyl})-amino)anthraquinone was attached using the method employed in the synthesis of the A conjugates.

6.2.4 Spectral Analysis of the Peptides and their Respective Conjugates

The peptides and their respective conjugates were analysed using ^{1}HNMR and Mass Spectrometry. NMR analysis was performed in *d*-DMSO (100%).

Peptide 1

δ_H (1.25; Ala β) (1.35; Ala β) (1.5; Arg ,Lys β, χ, Lys δ) (2.7; Lys ε) (2.8; Cys β) (3.1; Arg δ) (3.9; Ala α) (4.2; Ala α) (4.3; Lys α) (4.4; Cys) (7.0-7.2 Arg guanidinium) (7.6; Arg side-chain (SC) NH) (7.8; Lys NH (SC)) (8.0; Ala, Cys) (8.1-8.3 Arg, Lys NH); m/z $(M + H)^+$ 548

Conjugate 1A

δ_H (1.2; Ala β) (1.5-1.6; Arg, Lys β, χ, Lys δ) (2.4; linker (L)) (2.75; Lys ε) (2.8; Cys β, L) (3.1; Arg δ, L) (4.0-4.5; Ala, Arg, Cys, Lys α) (7.2-7.6; Arg guanidinium) (7.5; Arg NH (SC)) (7.7; Lys NH (SC)) (7.9-8.5; Ala, Arg, Cys, Lys NH, ArCH) (9.7; NH); m/z $(M + H)^+$ 907

Peptide 2

δ_H (1.2; Ala β) (1.35; Ala β) (1.5-1.8; Arg ,Lys β, χ, Lys δ) (2.7; Lys ε) (2.8; Cys β) (3.1; Arg δ) (3.9; Ala α) (4.2; Ala α) (4.3-4.5; Arg, Cys, Lys α) (7.0-7.2 Arg guanidinium) (7.6; Arg (SC) NH) (7.8; Lys NH (SC)) (8.1-8.3; Ala, Cys, Lys NH); m/z $(M + H)^+$ 547

Conjugate 2A

δ_H (1.2; Ala β) (1.35; Ala β) (1.5-1.8; Arg, Lys β, χ, Lys δ) (2.3; L) (2.75; Lys ε) (2.8; Cys β, L) (3.1; Arg δ, L) (3.4; Ala α) (4.25; Ala α) (4.3-4.4; Arg, Cys, Lys α) (7.0-7.3; Arg guanidinium) (7.5; Arg NH (SC)) (7.7; Lys NH (SC)) (7.9-8.5; Ala, Arg, Cys, Lys NH, Ar CH) (9.6; NH); m/z $(M + H)^+$ 895

Peptide 3

δ_H (1.1; Ala β) (1.25; Ala β) (1.5-1.7; Arg, Lys β, χ, Lys δ) (2.75; Lys ε) (3.1; Arg, δ, Ser β) (3.9; Ala α) (4.15; Ala, α) (4.2-4.4; Arg, Lys, Ser α) (7.0-7.2; Arg guanidinium) (7.6; Arg NH (SC)) (7.8; Lys NH (SC)) (7.9; Ala NH) (8.0-8.5; Ala, Arg, Lys, Ser NH); m/z $(M + H)^+$ 531

Conjugate 3A

δ_H (1.2; Ala β) (1.35; Ala β) (1.5-1.7; Arg, Lys β, χ, Lys δ) (2.3; L) (2.75; Lys ε) (2.8; L) (3.1; Arg, δ, Ser β, L) (3.9; Ala α) (4.2-4.4; Ala, Arg, Lys, Ser α) (7.0-7.3; Arg guanidinium) (7.5; Arg NH (SC)) (7.8; Lys NH (SC)) (79; Ala NH) (8.0-8.5; Ala, Arg, Lys, Ser NH; Ar) (9.5; NH); m/z $(M + H)^+$ 879

Peptide 4

δ_H (1.1; Ala β) (1.2; Ala β) (1.3-1.7; Arg ,Lys β, χ, Lys δ) (2.6; Asn β) (2.75; Lys ε) (2.8; Cys β) (3.1; Arg δ) (3.9; Ala α) (4.15; Ala α) (4.2; Arg, Lys α) (4.3; Cys) (4.4; Asn) (7.1-7.3 Arg guanidinium) (7.6; Arg NH (SC)) (7.8; Lys NH (SC)) (7.9; Ala NH) (8.1-8.4; Ala, Asn, Cys, Lys NH); m/z $(M + H)^+$ 661

Conjugate 4A

δ_H (1.2; Ala β) (1.3-1.6; Arg, Lys β, χ, Lys δ) (2.4; L) (2.6; Asn β) (2.75; Lys ε) (2.8; Cys β, L) (3.1; Arg δ, L) (3.4; L) (4.05; Ala α) (4.2; Ala α) (4.3-4.4; Arg, Cys, Lys α) (4.45; Asn α) (7.0-7.4; Arg guanidinium) (7.5; Arg NH (SC)) (7.6;

Lys NH (SC)) (7.9; Ala NH) (8.0-8.3; Ala, Arg, Cys, Lys, Ar CH) (9.75; NH); m/z $(M + H)^+$ 1009

Conjugate 4D

δ_H (1.15; Ala β) (1.2; Ala β) (1.4-1.6; Arg, Lys β, χ, Lys δ) (2.3-2.4; L) (2.5; Asn β) (2.7-2.8; Cys β, Lys ε, L) (2.9; L) (3.1; Arg δ, L) (3.3; L) (4.1; Ala α) (4.2-4.4; Ala, Arg, Cys, Lys α, L) (4.5; Asn α) (7.1-7.4; Arg guanidinium) (7.6; Arg NH (SC)) (7.7; Lys NH (SC)) (7.8; Ala NH) (7.9-8.3; Ala, Arg, Asn, Cys, Lys, Ar CH) (9.75; NH); m/z $(M + H)^+$ 1137

Conjugate 4F

δ_H (1.1; Ala β) (1.3-1.6; Arg, Lys β, χ, Lys δ) (2.5; Asn β, L) (2.7-2.8; Cys β, Lys ε) (2.9; L) (3.1; Arg δ, L) (3.8; L) (4.1; Ala α) (4.2-4.4; Ala, Arg, Cys, Lys α) (4.5; Asn α) (7.0-7.5; Arg guanidinium) (7.5; Arg NH (SC)) (7.8; Lys NH (SC)) (7.9; Ala NH) (7.9-8.3; Ala, Arg, Asn, Cys, Lys, Ar CH); m/z $(M + H)^+$ 1122

Conjugate 4H

δ_H (1.1; Ala β) (1.1; Ala β) (1.4-1.8; Arg, Lys β, χ, Lys δ) (2.6; Asn β, L) (2.8; Cys β, Lys ε) (3.1; Arg δ, L) (3.5; L) (3.8; Ala α,) (4.1; Ala α) (4.2-4.4; Arg, Cys, Lys α) (4.5; Asn α) (7.1-7.3; Arg guanidinium) (7.5; Arg NH (SC)) (7.7; Lys NH (SC)) (7.8; Ala NH) (7.9-8.3; Ala, Arg, Asn, Cys, Lys, ArCH); m/z $(M + H)^+$ 1151

Peptide 5

δ_H (1.2; Ala β) (1.35; Ala β) (1.5-1.7; Arg ,Lys β, χ, Lys δ) (2.7-2.8; Cys β, Lys ε) (3.1; Arg δ) (3.9; Ala α) (4.2; Ala α) (4.3-4.5; Arg, Cys, Lys α) (7.0-7.4 Arg guanidinium) (7.7; Arg NH (SC)) (7.8; Lys NH (SC)) (7.9; Ala NH)(8.0-8.5; Ala, Arg, Cys, Lys NH); m/z $(M + H)^+$ 675

Conjugate 5A

δ_H (1.2; Ala β) (1.3-1.6; Arg, Lys β, χ, Lys δ) (2.4; L) (2.7-2.8; Cys β, Lys ε, L) (3.1; Arg δ, L) (3.4; L) (4.1-4.5; Ala, Arg, Cys, Lys α) (7.4-7.6; Arg guanidinium) (7.5; Arg NH (SC)) (7.6; Lys NH (SC)) (7.7-8.2; Ala, Arg, Cys, Lys NH, ArCH); m/z $(M + H)^+$ 1023

Conjugate 5D

δ_H (1.2; Ala β) (1.25; Ala β) (1.35-1.6; Arg, Lys β, χ, Lys δ) (2.3; L) (2.7-2.8; Cys β, Lys ε, L) (2.9; L) (3.1; Arg δ) (3.4; Ala α) (4.2; Ala, Arg, Lys α) (4.4-4.5; Ala, Cys, α, L) (7.0-7.3; Arg guanidinium) (7.6; Arg NH (SC)) (7.8; Lys NH (SC)) (7.9-8.5; Ala, Arg, Cys, Lys NH, Ar CH) (9.7; NH); m/z $(M + H)^+$ 1151

Peptide 6

δ_H (1.25; Ala β) (1.4; Ala β) (1.5-1.7; Arg ,Lys β, χ, Lys δ) (2.6; Asn β) (2.75; Lys ε) (2.8; Cys β) (3.0; Arg δ) (3.9; Ala α) (4.1; Ala α) (4.2; Arg, Lys α) (4.4; Cys) (4.5; Asn) (7.0-7.3 Arg guanidinium) (7.5; Arg NH (SC)) (7.8; Lys NH

(SC)) (7.9; Ala NH) (8.1; Ala, Arg, Lys NH) (8.3; Cys NH) (8.5; Asn NH); m z $(M + H)^+$ 817

Conjugate 6A

δ_H (1.2; Ala β) (1.5-1.7; Arg, Lys β, χ, Lys δ) (2.4; L) (2.6; Asn β) (2.8; Cys β,Lys ε) (3.1; Arg δ, L) (3.4; L) (4.1-4.3; Ala, Arg, Cys, Lys α) (4.6; Asn α) (7.0-7.3; Arg guanidinium) (7.5; Arg NH (SC)) (7.7; Lys NH (SC)) (7.9-8.3; Ala, Arg, Asn, Cys, Lys, ArCH); m/z $(M + H)^+$ 1165

Conjugate 6D

δ_H (1.2; Ala β) (1.3; Ala β) (1.5-1.7; Arg, Lys β, χ, Lys δ) (2.3-2.4; L) (2.6; Asn β) (2.7-2.8; Cys β, Lys ε, L) (2.9-3.1; Arg δ, L) (3.3; L) (4.1; Ala α) (4.2-4.4; Ala, Arg, Cys, Lys α, L) (4.5; Asn α) (7.1-7.3; Arg guanidinium) (7.6; Arg NH (SC)) (7.8; Lys NH (SC)) (8.0-8.3; Ala, Arg, Asn, Cys, Lys, ArCH) (9.7; NH); m/z $(M + H)^+$ 1293

Peptide 7

δ_H (1.2; Ala β) (1.3; Ala β) (1.3-1.6; Arg ,Lys β, χ, Lys δ) (2.7-2.8; Cys β, Lys ε) (3.1; Arg δ) (3.9; Ala α) (4.1; Ala α) (4.2; Arg, Lys α) (4.4; Cys α) (7.1-7.3 Arg guanidinium) (7.6; Arg NH (SC)) (7.75; Lys NH (SC)) (7.8; Ala NH) (8.0-8.5; Ala, Arg, Cys, Lys NH); m/z $(M + H)^+$ 831

Conjugate 7A

δ_H (1.2; Ala β) (1.4-1.6; Arg, Lys β, χ, Lys δ) (2.3-2.4; L) (2.7-2.8; Cys β, Lys ε, L) (3.1; Arg δ, L) (3.4; L) (4.1-4.5; Ala, Arg, Cys, Lys α) (7.1-7.3; Arg guanidinium) (7.6; Arg NH (SC)) (7.7-8.3; Lys NH (SC), Ala, Arg, Cys, Lys NH, ArCH); m/z $(M + H)^+$ 1179

Conjugate 7D

δ_H (1.2; Ala β) (1.25; Ala β) (1.4-1.6; Arg, Lys β, χ, Lys δ) (2.3; L) (2.7-2.8; Cys β, Lys ε, L) (2.9-3.1; Arg δ, L) (4.1-4.5; Ala, Arg, Cys, Lys α, L) (7.0-7.3; Arg guanidinium) (7.6; Arg NH (SC)) (7.7; Lys NH (SC)) (7.8-8.5; Ala, Arg, Cys, Lys NH, ArCH) (9.8; NH); m/z $(M + H)^+$ 1307

Conjugate 7F

δ_H (1.2; Ala β) (1.3; Ala β) (1.5-1.7; Arg, Lys β, χ, Lys δ) (2.6; L) (2.7-2.8; Cys β, Lys ε, L) (3.1; Arg δ, L) (3.9; Ala α) (4.2; Ala) (4.3; Arg, Lys α) (4.4; Cys α) (7.0-7.4; Arg guanidinium) (7.7; Arg NH (SC)) (7.9-8.2; Lys NH (SC), Ala, Arg, Cys, Lys NH, ArCH); m/z $(M + H)^+$ 1293

Conjugate 7H

δ_H (1.2; Ala β) (1.5-1.6; Arg, Lys β, χ, Lys δ) (2.2-2.5; L) (2.8; Cys β, Lys ε, L) (3.0; Arg δ, L) (3.5; L) (4.1-4.5; Ala, Arg, Cys, Lys α) (7.1-7.3; Arg guanidinium) (7.6; Arg NH (SC)) (7.7; Lys NH (SC)) (7.9; Ala NH) (8.0-8.3; Ala, Arg, Cys, Lys NH, ArCH) (9.7; NH); m/z $(M + H)^+$ 1322

Peptide 8

δ_H (1.2; Ala β) (1.25; Ala β) (1.35; Ala β) (1.4-1.6; Arg ,Lys β, χ, Lys δ) (2.75; Lys ε) (2.8; Cys β) (3.1; Arg δ) (3.9; Ala α) (4.2; Ala α) (4.3; Arg, Lys α) (4.4; Cys α) (7.0-7.3 Arg guanidinium) (7.5; Arg NH (SC)) (7.7; Lys NH (SC)) (7.8-8.4; Ala, Arg, Cys, Lys NH); m/z $(M + H)^+$ 689

Conjugate 8A

δ_H (1.25; Ala β) (1.4-1.6; Arg, Lys β, χ, Lys δ) (2.4; L) (2.7-2.8; Cys β, Lys ε, L) (3.1; Arg δ, L) (3.4; L) (4.1-4.5; Ala, Arg, Cys, Lys α) (7.2-7.4; Arg guanidinium) (7.5; Arg NH (SC)) (7.7-8.3; Lys NH (SC), Ala, Arg, Cys, Lys NH, ArCH) (9.8; H); m/z $(M + H)^+$ 1037

Conjugate 8D

δ_H (1.1; Ala β) (1.2; Ala β) (1.3-1.5; Arg, Lys β, χ, Lys δ) (2.3; L) (2.7-2.8; Cys β, Lys ε, L) (3.1; Arg δ, L) (3.4; L) (3.5; Ala α) (4.15; Ala) (4.2; Arg, Lys α) (4.3; Ala α) (4.4; Cys α) (7.0-7.3; Arg guanidinium) (7.5; Arg NH (SC)) (7.7-8.3; Lys NH (SC), Ala, Arg, Cys, Lys NH, ArCH); m/z $(M + H)^+$ 1165

6.4 DNA-Drug Interactions

All glass apparatus used in these studies were silanised with dichloromethylsilane, washed with methanol followed by water and dried at room temperature before

use. The volumetric glassware used was of grade A specification. All volumes less than 1.0ml were measured using Hamilton precision glass syringes. The water used was either freshly prepared double distilled or Millipore water.

Tris buffers (0.008M Tris hydrochloride; pH 7.2) with either sodium chloride concentrations of 0.5M or 0.05M were prepared. The former was used in the determination of the isosbestic points and the latter was used in the fluorescence and thermal denaturation studies. Stock peptide conjugate solutions were prepared in 1-2% v/v DMSO in the appropriate Tris buffer. The DNA solution was prepared by dissolving calf thymus DNA in each of the buffers to give a solution of approximately 2mg ml^{-1}. The concentration of DNA, expressed in terms of the molar concentration of DNA phosphate was determined at 260nm using $E = 6600M^{-1}cm^{-1}$. All solutions were protected from light and stored at 4°C.

6.4.1 Spectroscopic Studies

Calf thymus DNA in Tris buffer ($5 \times 10^{-3}M$) was added to eight 10ml volumetric flasks containing the peptide conjugate (1.5×10^{-5}) in the same buffer so that the DNA:drug concentration varied from 0 to 15:1. After each addition the solutions were made up to volume using buffer, gently shaken and left to stand for one minute. The spectra of the solutions were recorded superimposed using a Perkin Elmer UV/VIS Spectrophotometer against a buffer blank over the range of the maximum absorbance of the peptide conjugate in the visible region of the spectrum.

6.4.2 Fluorescence Studies

Aliquots (6 x 10µl, 5 x 20µl, 6 x 40µl, 6 x 100µl) of ethidium bromide solution (2 x 10^{-5}M) were added sequentially to a 3.0ml volume of each of the following solutions i) 2 x 10^{-5}M calf thymus DNA in Tris buffer, pH 7.2; ii) 2 x 10^{-5}M calf thymus DNA and 2 x 10^{-6}M of peptide conjugate in buffer; iii) 2 x 10^{-6}M peptide conjugate in buffer and iv) buffer. After each addition the solution was gently stirred and allowed to stand for one minute. The fluorescence intensity was then determined at 25°C in triplicate using a Perkin Elmer LS-5 Luminescence Spectrometer at $\lambda_{emission}$ 596nm ($\lambda_{excitation}$ 479nm). The mean fluorescence intensities were corrected for changes in volume and the above experiment was repeated twice.

6.4.3 Thermal Denaturation Studies

Calf thymus DNA (0.2ml; 4.6 x 10^{-3}M) in Tris buffer, pH 7.2 was added to an appropriate volume of peptide conjugate (9 x 10^{-6}M) in 3ml of the same buffer such that the DNA to drug ratio was 10:1. The volume was made up to 10ml with Millipore water. The changes in absorbance of the DNA-conjugate solution were recorded at 260nm as the temperature was raised from 60 to 105°C (0.5°Cmin^{-1}) in a Perkin Elmer Spectrophotometer 552 fitted with a temperature programmer. The above experiment was repeated three times for each conjugate.

6.5 Structural Analysis

6.5.1 NMR Spectroscopic Studies

Spectra were acquired on a Bruker AC 250 MHz spectrometer equipped with an Aspect 3000 computer. By use of standard methods a set of 2-dimensional spectra were accumulated in phase incrementation: Double Quantum Filtered Phase Sensitive COSY and NOESY – with mixing times in the range of 100-600ms. Data were accumulated with 16 transients and 4 dummy transients in 2048 data points in the second time domain (t_2) and in 1024 in the first one (t_1). Spectra were recorded in both DMSO and TFE.

6.5.2 Circular Dichroism Studies

The circular dichroism of the peptides was measured with a Jobin-Yvon CD6 spectrophotometer. All experiments were performed in the UV region (180-270nm) at 25°C, using 0.5mm path length quartz cells.

6.5.3 Molecular Modelling Studies

Studies were conducted on the peptides and their interaction with the DNA AP-1 site in an aqueous environment. The structures in each case were minimised using an RMS first derivative of 0.001kcal/mol, which was achieved typically with 5000 iterations using Insight and Discover software (Biosym Technologies, San Diego, CA, USA).

REFERENCES

1 Farmer, P. B., Walker, J. M. The Molecular Basis of Cancer. Croom Helm, London, 1985

2 Rak, J., Mitsuhashi, Y., Bayko, L., Filmus, J., Shirasawa, S., Sasazuki, T., Kerbel, R. S. Mutant *ras* Oncogenes Upregulate VEGF/VPF Expression: Implications for Induction and Inhibition of Tumour Angiogenesis. *Cancer Research* 1995, **55**, 4575

3 Vaupel, P. W. Oxygenation of Solid Tumours. In *Drug Resistance in Oncology* ; Edited by Teicher, B. A; 1993, 53

4 Jenkins, T. C., Parrick, J., Porssa, M. DNA-Binding Properties of Nitroarene Oligopeptides Designed as Hypoxia-Selective Agents. *Anti-Cancer Drug Design* 1994, **9**, 477

5 Yao, K., Xanthoudakis, S., Curran, T., O'Dwyer, P. J. Activation of AP-1 and of a Nuclear Redox Factor, Ref-1, in the Response of HT29 Colon Cancer Cells to Hypoxia. *Molecular and Cellular Biology* 1994, **14(9)**, 5997

6 Gatenby, R., Kessler, H., Rosenblum, J. S., Coia, L., Moldofsky, P. J., Hartz, W. H., Broder, G. Oxygen distribution in Squamous Cell Carcinoma Metastases and its Relationship to Outcome of Radiation Therapy. *Int. J. Radiation Oncology Biol. Phys* 1988, **14**, 831

7 Crooke, S. T., Prestayko, A. W. Cancer and Chemotherapy, Volume 1. New York Academic Press, 1980

8 Brown, J.M., Giaccia, A. J. Tumour Hypoxia: The Picture Has Changed in the 1990s. *Int. J. Radiat. Biol.* 1994, **65(1)**, 95

9 Coleman, C. N. Chemical Modification of Radiation and Chemotherapy. In: Cancer: Principles and Practice of Oncology. JB. Lippin; Philadelphia, 1989

10 Vogt, P. K., Bos, T. J. Jun: Oncogene and Transcription Factor. *Advances in Cancer Research* 1990, **55**, 1

11 Xanthoudakis, S., Miao, G., Weng, F., Pan, Y. E., Curran, T. A Repair Enzyme mediates Redox Activation of Fos-Jun DNA Binding Activity. *The EMBO Journal* 1992, **11(9)**, 3323

12 Nakabeppu, Y., Nathans, D. The Basic Region of Fos Mediates Specific DNA Binding. *The EMBO Journal* 1989, **8(12)**, 3833

13 Banyopadhyay, R. S., Phelan, M., Faller, D. V. Hypoxia Induces AP-1 Regulated Genes and AP-1 Transcription Fcator Binding in Human Endothelial and other Cell Types. *Biochimica et Biophysica Acta 1995*, **1264**, 72

14 Olive, M., Krylov, D., Echlin, D. R., Gardener, K., Taparowsky, E., Vinson, C. A Dominant Negative to Activation Protein-1 (AP-1) that Abolishes DNA Binding and Inhibits Oncogenesis. *J. Biological Chemistry 1997*, **272(30)**, 18586

15 Li, Y., Jaiswal, A. K. Regulation of Human NAD(P)H: Quinone Oxidoreductase Gene. *J. Biological Chemistry* 1992, **267(21)**, 15097

16 Yao, K., Hageboutros, A., Ford, P., O'Dwyer, P. J. Involvement of Activator Protein-1 and Nuclear Factor-*k*B Transcription Factors in the Control of the DT-Diaphorase Expression Induced by Mitomycin C Treatment. *Molecular Pharmacology* 1997, **51**, 422

17 Ellenberger, T. E., Brandl, C. J., Struhl, K., Harrison, S. C. The GCN4 Basic Region Leucine Zipper Binds DNA as a Dimer of Uninterrupted α Helices: Crystal Structure of the Protein-DNA Complex. *Cell* 1992, **71**, 1223

18 Cuenoud, B., Schepartz, A. Altered Specificity of DNA-Binding Proteins with Transition Metal Dimerisation Domains. *Science* 1993, **259**, 510

19 Kouzarides, T., Ziff, E. The Role of the Leucine Zipper in the Fos-Jun Interaction. Nature 1988, **336**, 646

20 Pabo, C. O., Sauer, R. T. Transcription Factors: Structural Families and Principles of DNA Recognition. *Annu. Rev. Biochem.* 1992, **61**, 1053

22 Vinson, C., Sigler, P. B., McKnight, S. L. Scissors-Grip Model for DNA Recognition by a Family of Leucine zipper Proteins. *Science* 1989, **246**, 911

21 O'Neil, K. T., Hoess, R. H., DeGrado, W. F. Design of DNA-binding Peptides Based on the Leucine Zipper Motif. *Science* 1990, **249**, 774

23 Kerppola, T. K., Curran, T. Transcription Factors Interactions: Basics on Zippers. *Current Opinion in Structural Biology* 1991, **1**, 71

24 Rauscher III, F. J., Voulalas, P. J., Franza Jr, B. R., Curran, T. Fos and Jun Bind Co-operatively to the AP-1 Site: Reconstitution in Vitro. *Genes and Development* 1989, **2**,1687

25 Abate, C., Patel, L., Rauscher III, F. J., Curran, T. Redox Regulation of Fos and Jun DNA-Binding Activity in Vitro. *Science* 1990, 249, 1157

26 Turner, R., Tjian, R. Leucine Repeats and an Adjacent DNA Binding Domain Mediate the Formation of Functional cFos-cJun Heterodimers. *Science* 1989, **243**, 1689

27 Brown, C. L., Harding, M. Synthetic α-Helical Peptides Incorporating Intercalators for DNA Recognition. *J. Molecular Recognition* 1994, **7**, 215

28 Gentz, R., Rauscher III, F. J., Abate, C., Curran, T. Parallel Association of Fos and Jun Leucine Zippers Juxtaposes DNA Binding Domains. Science 1989, **243**, 1695

29 Abate, C., Luk, D., Curran, T. A Ubiquitous nuclear Protein Stimulates the DNA-binding Activity of Fos and Jun Indirectly. *Cell Growth and Development* 1990, **1**, 455

30 Toledano, M. B., Leonard, W. J. Modulation of Transcription Factor NF-*k*B Binding Activity by Oxidation-Reduction in Vitro. *Proc. Natl. Acad. Sci. USA* 1991, **88**, 4328

31 Denny, W. A. DNA Intercalating Ligands as Anti-Tumour Cancer Drugs: Prospects for Future Design. *Anti-Cancer Design* 1989, **4(4)**, 241

32 Wilson, W. D., Jones, R. L. Intercalating Drugs: *DNA Binding and Molecular Pharmacology* 1981, **18**, 177

33 Baguley, B. C. DNA Intercalating Anti-tumour Agents. *Anti-Cancer Design* 1991, **6**, 1

34 Gale, E. F., Cundliffe, E., Reynolds, P. E., Richmond, M. H., Waring, M. J. The Molecular Basis of Antibiotic Action, 2nd ed.; John Wiley and Sons, London, 1972

35 Remers, W. A. The Chemistry of Antitumour Antibiotics, Volume 1; John Wiley and sons, New York, 1979

36 Bailey, P. D. An Introduction to Peptide Chemistry; John Wiley and sons, Chichester, 1990

37 Jones, J. Amino Acid and Peptide Synthesis; Oxford University Press, Oxford, 1992

38 Bodanszky, M. Principles of Peptide Synthesis, 2nd ed.; Princeton, New Jersey, 1993

39 Greene, T. W., Wuts, P. G. M. Protective Groups in Organic Synthesis, 2nd ed.; John Wiley and Sons, New York, 1991

40 Stewart, J. M., Young, J. D. Solid Phase Peptide Synthesis; WH Freeman and Company, San Francisco, 1969

41 Atherton, E., Sheppard, R. C. Solid Phase Peptide Synthesis, A practical Approach; Irl Press, Oxford, 1989

42 Kaiser, E., Colescott, R. L., Bossinger, C. D., Cook, P. I. Colour Test of

Free Terminal Amino Groups in the Solid-Phase Synthesis of Peptides. *Anal. Biochemistry* 1970, **34**, 595

43 Bedford, J., Hyde, C., Johnson, T., Jun, W., Owen, D., Quibell, M., Sheppard, R. C. *Int. J. Peptide Protein Res.* 1992, **40**, 300

44 Zubay, G. L. Biochemistry, 4th ed.; WC Brown Publishers, 1996

45 Scholtz, J. M., Qian, H., Robbins, V. H., Baldwin, R. L. The Energetics of Ion-Pair and Hydrogen-Bondong Interactions in a Helical Peptide. *Biochemistry* 1993, **32**, 9668

46 Sonnichsen, F. D., Van Eyk, J. E., Hodgees, R. S., Sykes, B. D. Effect of Trifluoroethanol on Protein Secondary Structure: A NMR and CD Study Using a Synthetic Actin Peptide. *Biochemistry* 1992, **31**, 8790

47 Ravi, A., Prasad, V., Balaram, P. Cyclic Peptide Disulfides. Solution and Solid State Conformation of a Disulfide-Bridged Peptide Helix. *J. Am. Chem. Soc.* 1983, **105**, 105

48 Jackson, D. Y., King, D. S., Chmielewski, J., Singh, S., Schultz, P. G. General Approach to the Synthesis of Short α-Helical Peptides. *J. Am. Chem. Soc.* 1991, **113**, 9391

49 Chorev, M., Roubini, E., McKee, R. L., Gibbons, S. W., Goldman, M. E., Caulfield, M. P., Rosenblatt, M. Cyclic Parathyroid Hormone Related Protein Antagonists: Lysine 13 to Aspartic Acid 17 [*i* to (*i* + 4)] Side Chain to Side Chain Lactamisation. *Biochemistry* 1991, **30**, 5968

50 Phelan, J. C., Skelton, N. J., Braisted, A. C., McDowell, R. S. A General Method for Constraining Short Peptides to an α-Helical Conformation. *J. Am. Chem. Soc* 1997, **119(3)**, 455

51 Plumbridge, T. W., Brown, J. R. The Interaction of Adriamycin and Adriamycin Analogues with Nucleic Acids in the B and A Conformations. *Biochemica et Biophysica Acta* 1979, **563**, 181

52 Plumbridge, T. W., Brown, J. R. Spectrophotometric and Fluorescence Polarisation Studies of the Binding of Ethidium, Daunomycin and Mepacrine to DNA and to Poly(I-C). *Biochemica et Biophysica Acta* 1977, **479**, 441

53 Laurence, D. J. R. A Study of the Adsorption of Dyes on Bovine Serum Albumin by the Method of Polarisation of Fluorescence. *Biochem. J.* 1952, **51**, 168

54 Scatchard, G. The Attractions of Proteins for Small Molecules and Ions. *ANN NY ACAD SCI* 1949, **51** 660

55 LePecq, J.-B., Paoletti, C. A Fluorescent Complex between Ethidium Bromide and Nucleic Acids. *J. Mol. Biol.* 1967, **27**, 87

56 Paoletti, J., LePecq, J. Resonance Energy Transfer Between Ethidium Bromide Molecules Bound to Nucleic Acids. *J. Mol. Biol* 1971, **59**, 43

57 Gandecha, B. M. Development of Potential Anti-Tumour Agents Based on a Consideration of the Mode of Action and Pharmacokinetics of Daunomycin and Adriamycin. PhD Thesis, Leicester Polytechnic, 1985

58 Zunino, F., Gambetta, R., Di Marco, A., Zaccara, A. Interaction of its Derivatives with DNA. *Biochemica et Biophysica Acta 1972*, **277**, 489, 1

59 Marmur, J., Doty, P. Determination of the Base Composition of DNA from its Thermal Denaturation Temperature. *J. Mol. Biol.* 1962, **5**, 109, 1

60 Waring, M. Variation of the Supercoils in Closed Circular DNA by Binding of Antibiotics and Drugs: Evidence for Molecular Models Involving Intercalation. *J. Mol. Biol.* 1970, **54**, 247

61 Revzin, A. Gel Electrophoresis Assays for DNA-Protein Interactions. *Biotechniques* 1989, **7(4)**, 346

62 Hassanain, H. H., Dai, W., Gupta, S. L. Enhanced Gel Mobility Shift Assay for DNA-Binding Factors. *Analytical Biochemistry* 1993, **213**, 162

63 Abraham, R. J., Fisher, J., Loftus, P. Introduction to NMR Spectroscopy; John Wiley and Sons, Chichester, 1988

64 Gunther, H. NMR Spectroscopy, 2nd ed.; John Wiley and Sons, Chichester, 1995

65 Roberts, G. C. K. NMR of Macromolecules, A Practical Approach; Oxford University Press, New York, 1993

66 Purdie, N., Brittain, H. G., Analytical Applications of Circular Dichroism; Elsevier, Amsterdam, 1994

67 Neidle, S., Jenkins, T. C. Molecular Modelling to Study DNA Intercalation by Anti-tumour Agents. *Methods in Enzymology* 1991, **203**, 433

68 Coste, J., Le-Nguyen, D., Castro, B. PyBOP: A New Peptide Coupling Reagent Devoid of Toxic By-product. *Tetrahedron Letters* 1990, **31(2)**, 205

69 Fields, G. B. Solid Phase Peptide Synthesis;. *Int J Pep Prot Res* 1990, **35**, 161

70 Tran, P. Anthrapyrazole Cysteinyl Peptides as Inhibitors of AP-1 Transcription Factor Binding. PhD Thesis, De Montfort University, 1998

71 Ruparelia, K. Personal Communications. De Montfort University

72 Panniwynk, Z. Personal Communications. De Montfort University

73 Slagle, J.D., Huang, T.-S., Franzus, B. Mechanism of the Triphenyl-phosphine-Tetrachloromethane-Alcohol Reaction: Pericyclic or Clustered Ion Pairs? *J. Org. Chem.* 1981, **46**, 3526

74 Sasaki, T., Minamoto, K., Itoh, H. Convenient Synthesis of some Purine 8,5'-Imino Cyclonucleosides. *J. Org. Chem.* 1978, **43(12)**, 2320

75 Neidle, S. Topics in Antibiotic Chemistry, 2nd ed.; Ed. Sammes, P. G.: Ellis Horwood, Chichester, 1978

76 Islam, S. A., Neidle, S., Gandecha, B. M., Partridge, M., Patterson, L. H., Brown, J. R. Comparative Computer Graphics and Solution Studies of the DNA Interaction of Substituted Anthraquinones Based on Doxorubicin and Mitoxantrone. *J. Med. Chem.* 1985, **28**, 857

77 Tanious, F. A., Jenkins, T. C., Neidle, S., Wilson, W. D. Substituent Position Dictates the Intercalative DNA-Binding Mode for Anthracene–9,10-dione Antitumour Drugs. *Biochemistry* 1992, **31**, 11632

78 Chen, K-X., Gresh, N., Pullman, B., Energetics and Stereochemistry of DNA Complexation with the Anti-tumour AT Specific Intercalators Tylarone and N-AMSA. *Nucleic Acids Res* 1988, **16(7)**, 3601

79 Bailly, F., Bailly, C., Helbecque, N., Pommery, N., Colson, P., Houssier, C., Henichart, J. P. Relationship Between DNA-Binding and Biological Activity of Anilinoacridine Derivatives Containing the Nucleic Acid-Binding Unit SPKK. *Anti-Cancer Drug Design* 1992, **7**, 83

80 Saudek, V., Pasley, H. S., Gibson, T., Gausepohl, H., Frank, R., Pastore, A. Solution Structure of the Basic Region from the Transcriptional Activator GCN4. *Biochemistry* 199130, 1310

81 Talanian, R. V., McKnight, C. J., Kim, P. S. Sequence-Specific DNA Binding by a Short Peptide Dimer. *Science* 1990, **249**, 769

82 Talanian, R. V., McKnight, C. J., Rutkowski, R., Kim, P. S. Minimum Length of a Sequence-Specific DNA Binding Peptide. *Biochemistry* 1992, **31**, 6871

83 MacPhee, C. E., Perugini, M. A., Sawyer, W. H., Howlett, G. J. Trifluoroethanol Induces the Self-association of Specific Amphipathic Peptides. *FEBS Letters* 1997, **416**, 265

84 Haris, P. Personal Communications, De Montfort University

85 Stryer, L. Biochemistry, 3rd ed.; W. H. Freeman and Company, New York, 1988

86 Bernier, J-L., Catteau, J-P. Design, Synthesis and DNA-Binding Capacity of a New Peptidic Bifunctional Intercalating Agent. *Biochem. J* 1981, **199**, 479

87 Wang, Y., Huang, L., Wright, S. C., Larrick, J. W. Doxorubicin and DNA Minor Groove-Binding Oligopeptide Conjugates as Anticancer Agents. *Gene* 1994, **149**, 63

88 Tabor, A. B. Synthesis of a Peptide-Intercalator Hybrid Based on the bZIP Motif from GCN4. *Tetrahedron* 1996, **52(6)**, 2229

89 Takenaka, S., Sato, H., Itakura, Y., Kondo, H., Takagi, M. Construction of a Dimeric DNA –Binding Peptide Model by Peptide-Anthraquinone Conjugation. *Int. J. Peptide Protein Res.* 1996, **48**, 397